STUDIES ON THE INTRACELLULAR STATUS AND LOCALIZATION OF MOUSE PANCREAS RIBONUCLEASES

by

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A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biological Chemistry

University of Utah

October, 1958

This Thesis for the Ph.D. degree

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ACKNOWLEDGEMENTS

I wish to express my deep gratitude and thanks to Dr. Sherman R. Dickman for his help and encouragement during my graduate studies in the Department of Biological Chemistry. His interest and enthusiasm were valuable stimuli during the course of this research.

The assistance and enthusiasm of Dr. Julian L. Van Lancker in carrying out the cell fractionation procedures is also deeply appreciated.

Special thanks to Miss Marjorie Riches for her invaluable aid in preparing this thesis.

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INTRODUCTION AND STATEMENT OF PROBLEM.

In recent years there has been a growing recognition of the role of integrated enzyme systems in cellular processes. The concept of the mitochondrion as an organized mosaic of several hundreds of enzymes linked together structurally as well as functionally and with unique operational principles is now well established in biochemical thinking. It is only in terms of large molecular aggregates and of enzymes bonded together in a precise pattern that it is at all possible to approach an understanding of the physiological processes of living cells. Thus these organized enzyme systems have provided the bridge between classical enzyme chemistry and cellular processes.

However, it becomes evident that there are inherent limitations when we rely on observations of highly purified individual enzymes. We must realize that by so doing we may lose sight of the role of this one specific enzyme in relation to other enzymatic events, and that in the process of isolation we may have changed the very nature of the enzyme as it originally existed in the cell. This brings us into the realms of nomenclature, semantics, and philosophy and raises the spectre of having to determine how and in what form enzymes preexisted in the cell.

But one may ask, "How are we to evaluate the intra-cellular status of an enzyme?" Like most problems in other scientific fields, problems in biochemistry do not have a spontaneous generation but arise and receive

their definition from the previous work of many people. Furthermore, the answers to many scientific questions often must await the development of new methods and techniques, whether these are borrowed from other fields or worked out specifically for the type of problem at hand. Such has been the case for the problem of this thesis which relates to the intra-cellular localization and status of the ribonuclease enzyme system in the acinar cell of the pancreas.

Thus, in designing experiments which seek to reveal the possible function and status of an intra-cellular enzyme, one can be guided by the fact that today at least four observational techniques are available. These "techniques" may be outlined as follows: 1. The presence of an enzyme (ribonuclease) or system of enzymes in mouse pancreas (1) which offer great advantages due to their stability, resistance to loss of enzymatic activity over a wide range of pH, an apparent lack of a co-factor requirement, and an extremely sensitive method of quantitation. 2. The existence of practical, rapid, and reproducible methods for the separation of the various formed elements of the cell, relatively free from each other and the soluble cytoplasmic constituents. 3. The third and possibly the most important technique is the chromatographic separation of mouse pancreatic "nuclease" activity into several related forms of the enzyme possessing unimpaired activity (1). This technique has, in fact, two distinct advantages, one to separate a system of closely related molecular species, and secondly, treatment with the column adsorbant may be used to remove much unwanted material from the enzyme solution.

And finally, 4., the rate of synthesis and turnover of proteins is very high in the pancreas compared to other tissues. Hence by inducing physiological changes in the tissue by means of varying periods of starvation and by stimulating the tissue to secrete its digestive enzymes one may selectively analyze various enzymes at any given point in the synthetic and secretory cycle of the cell. In fact, these induced changes in the pancreas are not limited to the living animal, but nave been shown to take place in the intact mouse pancreas *in vitro* (2), thus making it possible to study *in vitro* synthesis and secretion of an animal enzyme by means of enzymological techniques.

HISTORICAL REVIEW.

The biochemical problem selected for this thesis was that of the intracellular status and localization of the enzyme ribonuclease in the secretory cell of the pancreas. Historically, the problem has a three-fold development as related to the following chemical and biological studies: 1) Enzymatic studies on mammalian nucleases; 2) The separation of the formed elements of the cell into distinct fractions of functionally homogeneous particles; and 3) Studies of the physiological changes in the acinar pancreas induced by starvation and hormonal and neural stimulation. The physiological studies are somewhat contemporary and began rather early; the development of the first two has been within the last fifteen years.

Mammalain "Nucleases"

There is increasing evidence for the presence of numerous different specific and non-specific nucleases in mammalian tissues which are able to catalyze the cleavage of the internucleotide linkages of ribonucleic acid (3,4). The degrees of specificity of these enzymes of nucleic acid metabolism show widely different ranges. Many of these enzymes catalyze reactions not only of natural substrates, but also of analogous substances. At least two enzymes, beef pancreatic ribonuclease and spleen phosphodiesterase, catalyze transfer reactions in addition to hydrolysis (5,6). Both cyclic nucleotides and diesters of 3'-nucleotides serve as substrates for the transfer. Acceptors for the phosphate include the primary alcohol

groups of nucleosides, nucleoside cyclic phosphates, and simple alcohols. Simple nucleotides do not serve as acceptors. The products of transfer reactions, including dinucleoside monophosphates and the so-called cyclic dinucleotides, can also serve as acceptors, and by repeated transfer reactions of cyclic nucleotides small polymers have been built up.

The number of different enzymatic mechanisms in the hydrolytic cleavage of nucleic acids has become too large to fit into the original system of nomenclature proposed by Levene and Medigreceanu (7). The present terminology is therefore somewhat arbitrary because some original designations are now applied with a modified meaning. Levene and Medigreceanu introduced the collective term "nucleases" for all enzymes involved in the metabolism of nucleic acid or their degradation products or precursors. In contrast to this broad application of the term "nucleases", the terms "ribonucleases" and "deoxyribonucleases" - introduced by Dubos and Thompson (8) and by Kunitz (9), respectively - were usually limited to the designation of enzymes which catalyze the cleavage of the phosphoric ester bonds interlinking the nucleotide groups of nucleic acids or polynucleotides. However, since the isolation of crystalline pancreatic ribonuclease by Kunitz (10) in 1940, the presence of enzymes having the ability to hydrolyze high polymer RNA has been reported for many plant and animal sources. Through the use of many different assay methods and varying nomenclature, considerable confusion has arisen as to the action of these enzymes and their specificity. Schlamowitz and Garner (11) list "ribonuclease", "ribonucleodepolymerase", "polynucleotidase" and "nucleotidease" as names used to specify enzymes capable of degrading RNA. They in turn introduced the

term "nuclease activity" as indicative of the presence of enzymes which promote the hydrolysis of RNA with the formation of inorganic phosphate. In addition to these, Zittle and Reading (12) first reported on an enzyme different from pancreatic ribonuclease which they called "non-specific phosphodiesterase", and which was capable of splitting RNA.

There is now substantial unanimity (13,14), that in ribonucleic acids the nucleotides are connected predominantly, even if not exclusively, by phosphate bridges connecting position 5 in the ribose of one nucleotide to position 3 in the ribose of the next. From the available evidence it is probable, that ribonuclease splits only this diesterified phosphate between the purine or pyrimidine nucleotides, giving rise to mononucleotides, cyclic mononucleotides, and mixtures of di- to oligonucleotides. Ribonuclease may, therefore, be considered to be a phosphodiesterase. Differences arise both because of a specificity or lack of specificity for purine or pyrimidine internucleotide ester bonds or because some preparations of ribonuclease break the link connecting the phosphate and position 5 of a ribose, while others break it on the other side between the phosphate and the 3 position. There is no evidence that ribonuclease can break it in both positions nor that any enzyme can act as phosphomonoesterases as well and release free inorganic phosphate.

Therefore, in this thesis ribonuclease is defined as any enzyme acting on RNA to split diester phosphate links, giving rise to mixtures of mono- and oligonucleotides which are soluble in acid-alcohol. Since it is quite possible that several ribonucleases with varying nucleotide specificity occur in a tissue, the term "tissue ribonuclease activity"

will be used with a direct reference to the tissue involved.

Only a few of the known enzymes involved in nucleic acid metabolism have been thoroughly investigated, and as yet, only two have been obtained in crystalline form (3). The bovine pancreatic enzyme first crystallized by Kunitz (10) is unquestionably the best known of the ribonucleases. This pancreatic enzyme has been extensively investigated in recent years, and the properties, action, and structure of the crystalline bovine enzyme are moderately well known (3,4,11). To date, the available evidence indicates that, in its action on yeast RNA (pH optimum, ca. 7.7), crystalline beef pancreatic ribonuclease is specifically adapted to the hydrolysis of bonds linking the phosphoryl group of a pyrimidine nucleoside -3'-phosphate to the 5'-hydroxyl of an adjacent purine or pyrimidine nucleoside. However, in addition to the now classical crystalline enzyme of Kunitz, numerous observations made with tissue homogenates or crude extracts indicate that ribonucleases differing markedly in their properties from the digestive one of the pancreas and from each other occur in various cells. The presence in spleen of another specific polynuclease (15,16,17) is one such example. This spleen enzyme resembles pancreatic ribonuclease in many of its properties but has a pH optimum of 6.5. In addition, the pH optima for the action of several other diverse tissue ribonucleases also vary, values of 4.5, 6.0, 7.8, and 7.0 having been obtained for calf thymus (15,18), calf spleen (15), rat liver (19), rat kidney (20), and chick erythrocytes (21). The heat lability of the various preparations also vary, as do the effects of electrolytes on enzymatic activity (15,19).

The ribonuclease picture is further complicated by the existence of different enzymes in the same tissue. The presence and intracellular distribution in rat liver of two ribonucleases differing in their properties has been reported (19,22,23,24), as well as in various tissues of mouse and rat (25). In rat liver, for example, of the two ribonucleases demonstrated, the one actively maximally at pH 7.8 to 8.0 (alkaline ribonuclease) appears to be similar to, or possibly identical with, crystalline beef pancreatic RNase, whereas the second, active maximally at pH 5.8 to 6.0 (acid RNase), is quite different in its properties (18,23,26,27).

Clear evidence for the existence of a number of different ribonucleases and for the nature of their catalytic activity has been obtained by Hilmoe and Heppel on fractionated extracts of spleen (16). In addition to the specific polynuclease of Kaplan and Heppel mentioned above, they studied the action of spleen extracts not only on RNA, but also on the limit polynucleotides obtained by exhaustive hydrolysis of RNA with crystalline ribonuclease as well as on some simple synthetic nucleotide-P-esters of well defined structure. Hilmoe and Heppel thus prepared from these spleen extracts a ribonuclease which was free from phosphomonoesterases and which hydrolyzed the limit polynucleotide fraction of RNA approximately four times faster than RNA itself. The pH optimum for this enzyme was at pH 6.6, and in contrast to the heat stability of the beef pancreatic enzyme, the spleen ribonuclease was rapidly inactivated at 60°.

Martin and Porter (28) were the first to suggest, on the basis of their chromatographic analyses, that two distinct proteins possessing ribonuclease activity exist in beef pancreas. In fact, subsequent

studies on the chromatographic behavior of pancreatic ribonuclease, as well as the partial degradation by specific proteases, indicate that there may be several different forms of the enzyme all possessing unchanged enzymatic activity (29,30,31).

In addition, crystalline preparations of bovine pancreatic ribonuclease have been found to vary considerably in chromatographic homogeneity; some samples contained over 90 per cent of the main component (ribonuclease A), whereas others were less pure. It has also been noted by Moore and Stein (32) that when chromatographically homogeneous ribonuclease A is stored in the lyophilized state at 0° for 16 months or more, small amounts of a faster moving component appear. It thus cannot be stated whether or not the inhomogeneity of some crystalline preparations of bovine ribonuclease is a result of insufficient purification or arises during storage of an initially homogeneous material. There can be little doubt, however, that chromatographically homogeneous ribonuclease A represents predominately a single molecular species, inasmuch as Hirs, Stein, and Moore (33) have been able to deduce a sufficiently detailed structural formula for the enzyme to make it extremely improbable that a mixture of proteins was being investigated.

In addition to the enzymes mentioned above, "non-specific" phosphodiesterases have been demonstrated in extracts of the intestinal mucosa (34) and in the kidney (35).

A peculiar specificity of action has been found with an enzyme from guinea pig liver nuclei (36). This enzyme splits ordinary dinucleotide bonds to form 3'-phosphate esters. When it is incubated with cyclic

nucleotides as substrates, on the other hand, it liberates exclusively 2' esters. It is not certain, however, that a single enzyme catalyzes the two activities. In contrast, the only two other preparations which have been found to open cyclic nucleotides are those from pancreas and spleen.

In summation, therefore, these findings, together with the always possible and almost probable presence of zymogens and inhibitors in tissues, make the comparison of the ribonuclease content of various tissues, when assayed under the same conditions, extremely unreliable. It would appear that each tissue ribonuclease (as with all enzymes) should be studied as an individual entity and that generalizations based on one isolated example are extremely hazardous (36).

Separation of the formed elements of the cell - Much of our knowledge of the intra-cellular localization of enzymes has been the result of the study of preparations of particular cell structures obtained by differential centrifugation. By subjecting cell suspensions to conditions of high shear, it is possible to disrupt the cell without destroying the integrity of the nucleus or cytoplasmic particles. The various structural components of such a homogenate then sediment in the centrifuge at different rates, primarily owing to a difference in size, and can be separated by a series of runs, usually at increasing speeds. By resuspending the centrifuged pellets and recentrifuging, a number of fairly homogeneous fractions can be obtained.

Until the past year, however, there were few reports in the literature dealing with the fractionation of pancreatic tissue. For example, Claude

isolated microsomes and secretory granules from pancreas homogenized in saline and found them similar in many respects to those isolated from liver (37). Lang and Siebert (38) isolated nuclei from pancreatic homogenates (hog) prepared in 40 per cent sucrose solutions and, in collaboration with Fisher, tested nuclear and cytoplasmic fractions for proteolytic activity (39). Using the same technique, Siebert et al. (40) fractionated and tested the fractions for their effect on blood pressure. Hokin (41) separated zymogen granules and microsomes from dog pancreas and investigated the RNA content and proteolytic activity of these fractions. Petermann mentioned that "macromolecular nucleoprotein particles" similar to those found in liver and spleen homogenates (42,43) have been noted in ultracentrifugal analyses of pancreas preparations (44).

More recently, however, Siekevitz and Palade have extended their biochemical and morphological studies of liver particulate fractions to studies on similar fractions of guinea pig pancreas. The results of their initial study (45) indicate that as in liver, pancreatic microsomes are also fragments of the endoplasmic reticulum derived almost exclusively from the rough surfaced parts of the latter. Except for a few minor differences, in fact, the response of pancreatic microsomes to various reagents and experimental procedures is similar to that of hepatic microsomes. At variance with the results obtained in the fractionation of liver, however, postmicrosomal fractions which consist primarily of small, dense particles of nucleoprotein were obtained from pancreas homogenates. Subsequent work by these same authors (46,47) has been concerned with more elaborate studies on ribonuclease and trypsin-

activatable protease activities and morphological changes in pancreas particulates under conditions of starvation and feeding. In addition, similar studies have been recently reported by Barton and Laird (48,49) involving the pancreatic microsomes of pilocarpine treated rats and the changes in the intracellular distribution of amylase during a pilocarpine-induced secretory cycle in rat pancreas.

As a result of the work of Schneider, Claude, Hogeboom, Dounce and others, a more or less standard scheme of fractionation has been adopted; and, as a result, a mass of data has become available on the intracellular distribution of an impressive array of enzymes in a number of tissues. These data, particularly those involving ribonuclease, will be considered in greater detail in the discussion section of this thesis.

Physiological changes in the pancreas. The secretory cycle.

The foundation of our knowledge of the secretory process was laid by R. Heidenhain in 1868 (50). He was the first to recognize that the glandular cells of the pancreas and salivary glands manifest a series of internal changes which may be regarded as a secretory cycle. In the following half century, Heidenhain, Langley, and others performed a great number of histophysiological experiments in which they recognized the presence of characteristic granules in the apical region of the exocrine or acinar cells and demonstrated, in addition, that the granular population varies during the secretory cycle of the organ, reaching a minimum after feeding when the digestive enzymes produced by the gland appear in its ducts and finally in the intestine (51,52,53,54,55).

This secretory response has been demonstrated by numerous workers (56,57,58,59,60,61) to be under both chemical and nervous control. The chemical phase in particular, has been shown to be due to the release into the portal blood of one or more hormones from the intestinal mucosa which then reach the pancreas via the systemic circulation. Thus, the presence of secretagogues or acid in the upper duodenum results in the liberation into the circulation of a protein hormone, secretin, which stimulates the flow of a watery, highly alkaline juice, nearly devoid of enzymes. The discovery of secretin by Bayliss and Starling in 1902 is of particular historical interest in that it represents the earliest well-studied endocrine regulated mechanism. The word "hormone" was coined shortly thereafter to describe this material. In contrast, however, nearly five decades were to pass before the discovery of a second pancreas stimulating hormone, pancreozymin, an agent producing the same effects as vagal stimulation; the flow of a juice rich in enzymes. A general survey of the various agents (pilocarpine, pancreozymin, etc.) capable of inducing enzyme secretion with the simultaneous reduction in the number of apical granules, can be found in reference 56 of this thesis.

Although the histological and physiological methods employed fifty or sixty years ago were far from perfect, facts of primary importance were established in these investigations. The crude results obtained by the earlier investigators have been confirmed in our time by workers using more refined methods of research. Thus the series of changes known as the secretory cycle may be described briefly as follows:

1) During the period of secretion the amount of colloidal material present in the glandular cells in the form of secretory granules, as well as the amount of enzymes, gradually diminishes. 2) The histological picture observed in the gland can be correlated with the concentration of enzymes in the granular cells, viz., the more granules that are discharged from the tissue, the richer in enzymes is the secretion produced. 3) The depleted stores of secretory granules and of enzymes are replenished in the cells of the gland during the resting phase. The initially small number of granules gradually increases. Finally they fill the cell, which now has the familiar appearance of a "resting" cell, with diminished protoplasmic basal borders and a flattened nucleus depressed to the base of the cell. The word "resting" must be taken to mean "not actively secreting". The glandular cell, strictly speaking, does not rest when it is building up the chemically very complex precursors of its secretion (including enzymes), but the type of work that it does is changed.

EXPERIMENTAL

I. Materials.

In general, all of the inorganic chemicals, e.g. those used for the preparation of the buffer solutions, were analytical grade. All solutions and enzymatic reactions were routinely made up in, or carried out in demineralized water, respectively. This contained less than 1 part per million (expressed as NaCl) of ionic material and was prepared by passing distilled water through a mixed bed of equal parts of IRH - 120 and IR - 460 deionizing resin.

II. Analytical Methods.

In the course of these studies certain techniques were employed routinely. It is the purpose of this section to describe these techniques. In several cases, special methods or alterations of the routine procedures were used. These will be described in connection with the experiments or procedures in which they were employed.

All absorbancy readings in the ultraviolet region were made on the Beckman Model DU spectrophotometer.

A. Determination of Ribonuclease activity.

Enzymatic hydrolysis of polymerized ribose nucleic acid was determined by a spectrophotometric assay developed in this laboratory (62).

Buffer. Ribonuclease activity was routinely assayed at both pH 5.0 and pH 7.5. Analyses were carried out at pH 5.0 using a 0.2 M acetate buffer containing 0.01% gelatin and at 7.5 using 0.05 M tris buffer containing 0.2 M NaCl and 0.01% gelatin.

Substrate. Yeast nucleic acid was purified according to the method of Vischer and Chargaff (63). Commercial nucleic acid was dissolved in ammonical water at pH 6.2 and filtered to remove the insolubles. The nucleic acid was then precipitated with acidic alcohol, filtered and the precipitate washed with alcohol. The solid material was redissolved and precipitated once more as described above. The washed precipitate was dialyzed for 24 hours against ice-cold distilled water. The RNA was collected by centrifugation, washed successively with 66, 98, and 100% alcohol, and finally with ether and dried in vacuo over phosphorus pentoxide.

A 0.3% (w/v) solution of the purified substrate was used, the pH being adjusted to 5.0 or 7.0 (for the 7.5 assay) depending upon the pH at which the assay was to be carried out.

Standard enzyme. A stock solution of crystalline bovine ribonuclease (Armour), containing 1 mg./ml. was prepared in the appropriate buffer and frozen. This stock solution was routinely diluted with the acetate buffer to give a 1.0 γ/ml. from which various aliquots were taken for the pH 5.0 assay. At pH 7.5 the stock solution was diluted to 0.01 γ/ml. and aliquots taken as in the pH 5.0 assay.

Precipitating reagent. The reagent used for stopping the enzymatic reaction and for precipitating the undigested nucleic acid was prepared

by mixing two volumes of tertiary butyl alcohol with one volume of glacial acetic acid.

Assay procedure.

Aliquots of a suitable dilution of the enzyme solution were transferred to 140 x 10 mm. thick walled ignition tubes and the individual samples diluted to 0.5 ml. with the corresponding buffer. These solutions were then brought to 37° in a constant temperature bath. 0.5 ml. of the substrate solution, also at bath temperature, was added to each tube with mixing by gently twirling the tube. When a series of reactions were run, it was convenient to add the substrate at 15 or 30 second intervals to the individual test tubes. At the end of a 10 minute incubation period the reaction was stopped by adding 3.0 ml. of the precipitating reagent, in the same order as the substrate addition. After stopping the reaction, the tubes were allowed to stand for 30 minutes at room temperature to coagulate the precipitated nucleic acids, and then centrifuged in the cold at 1,500 rpm. for 30 minutes. After removal from the centrifuge, 1.0 ml. of the clear supernatant was removed, diluted to 6.0 ml. with distilled water and the absorbancy determined at 260 mμ. Controls containing substrate and buffer alone, as well as crystalline ribonuclease standards were run in all cases. "Dead enzyme control values" were determined by adding the precipitating reagent immediately prior to substrate addition. The optical density of the reaction supernatants, when corrected by subtracting the absorbancy of the controls, gives a measure of the extent of hydrolysis of the nucleic acid substrate. A unit of enzyme activity was defined as that amount which caused an absorbancy increment of 0.100 in the final diluted sample.

B. Determination of amylase activity.

Amylatic activity was determined by a slight modification of Somogyi's original method (64). This procedure is based on the change in the iodine-staining properties of the starch substrate during enzymatic hydrolysis. Although a large number of valuable methods have been described for the assay of amylase, the one described here has proven to be extremely sensitive, simple, reliable, and very rapid.

Starch solution. A solution containing 75 mg. of soluble starch and 250 mg. NaCl per 100 ml. was prepared by gentle heating followed by redilution of the cooled solution to the initial volume in a volumetric flask.

Iodine solution. 10 ml. of a 0.1 M aqueous iodine solution was diluted to 500 ml. with a 2% KI solution.

Procedure. 1.0 ml. of properly diluted enzyme was incubated at 37° with 4.0 ml. of the substrate solution. In all cases the enzyme dilutions were carried out in 0.5 M NaCl. At various intervals 1.0 ml. aliquots were removed from the reaction mixture and transferred to 0.7 x 7.5 cm. test tubes containing 0.5 ml. of the iodine solution. These were mixed and observed with a point light source using a 100 watt bulb. The end point is reached when the red-brown color of erythrodextrin is seen with a tint of purple. A calibration curve is established with the time interval to reach the end point as a function of reciprocal concentration of the enzyme. Best results are obtained when the end point is reached in 30 min.; accurate to 60 min.

Definition of unit. The enzyme unit is defined as that amount of

enzyme which will catalyze the hydrolysis of 3.0 mg. of starch in one minute at 37° in the presence of 0.5 M NaCl.

C. Measurement of proteolysis.

The digestion of urea-denatured hemoglobin was followed by the method of Anson (65). A stock solution of substrate was prepared by mixing seven parts of a 2% solution of urea-denatured hemoglobin and three parts of a 0.1 M KH_2PO_4 buffer solution to give a final pH of 7.5. 5.0 ml. of the substrate is added to a series of 175 x 15 mm. test tubes and brought to 37° in a constant temperature bath. 1.0 ml. of a proper dilution of the enzyme, also at bath temperature, was added to each tube with mixing. At the end of a 15 minute incubation period these were quickly mixed with 10 ml. of 0.3 M trichloroacetic acid solution and incubated for 30 minutes at 37° to coagulate the precipitate. At the end of this period, the suspensions were filtered through Whatman No. 3 filter paper and the absorbancy of the filtrates determined at 280 mμ in a Beckman Model DU spectrophotometer. Controls containing enzyme alone and substrate alone were run in each experiment. The absorbancy of the filtrates, gives a measure of the tyrosine and tryptophan liberated during proteolysis.

D. Estimation of ninhydrin positive material.

Amino acid and protein components were measured by the quantitative ninhydrin method of Cocking and Yemm (66). The reaction occurs stoichiometrically with quantitative yields of dioxohydrindylidene-dioxhydrindamine (DYDA), the probable end product of the reaction, and has a number of advantages over the other ninhydrin methods.

The method may be summarized as follows:

1.0 ml. of an appropriate dilution of the solution to be assayed was mixed with 0.5 ml. of 0.2 M citrate buffer at pH 5.0. The ninhydrin in methyl cellosolve (1.0 ml., 2% v/v) were added to this solution either separately or as a single solution. Separately these solutions are stable for at least one month, mixed for at least one week. The well mixed solution was heated for 20 minutes at 100° and cooled for 5 minutes in running tap water. The boiling point of the water-methyl cellosolve mixture is greater than 100°, and using tubes stoppered with a glass marble, evaporation losses during the heating period were negligible. The solution was read directly in a Coleman Junior spectrophotometer at 570 mμ. The color was quite stable for at least 1 hour at room temperature.

E. Determination of "Purine Ribonuclease" activity.

Measurement of the specific hydrolysis of secondary phosphate esters of purine nucleoside 3'-phosphates was based on the hydrolysis of nucleic acid "core". The core was prepared by exhaustive digestion of yeast nucleic acid with crystalline bovine pancreatic ribonuclease. 25 grams of yeast sodium nucleate (Schwarz Laboratories, Inc.) were dissolved in water, NaOH added to adjust to pH 7.5, and the final volume adjusted to 250 ml. 10 mg. of crystalline ribonuclease (Armour Laboratories) was added for each 100 ml. of ribonucleic acid solution and the mixture was incubated at 37° until there was no further liberation of acid groups as measured by change of pH. During the incubation, NaOH was added to maintain the solution at pH 7.5. Aliquots of this solution were dialyzed at 4° against cold distilled water for 72 hours. The dialyzed solution was concentrated by lyophilization, neutralized to pH 7.0, and made up to a

concentration of 17 mg./ml.

Spleen phosphodiesterase, a nonspecific diesterase, was prepared by the method of Hilmoe and Heppel (67). The spleen enzyme, crystalline beef ribonuclease, and mouse pancreas extracts were incubated with both ribonucleic acid and "core", and the extent of the degradation measured at pH 6.5 by the phosphodiesterase assay of Hilmoe and Heppel (14), and at pH 7.5 by a modification of the spectrophotometric assay of Dickman et al. (62).

The assay methods may be summarized as follows:

pH 6.5 Phosphodiesterase assay - The reaction mixture contained 0.04 ml. of 0.25 M sodium succinate-succinic acid buffer, pH 6.5, 0.02 ml. of 0.1 M $MgCl_2$, and 0.03 ml. of "core" solution containing 50 mg. per ml., enzyme, and water to make a total volume of 0.2 ml. Incubation was for 30 minutes at 37°, after which 0.2 ml. of 0.25 per cent uranium acetate in 2.5 per cent perchloric acid was added. The mixture was cooled in ice for 5 minutes and centrifuged. An aliquot (0.1 ml.) of the solution was removed, diluted 40-fold with distilled water, and its absorbancy measured at 260 mμ. A blank value corresponding to incubation without enzyme was corrected for the absorbancy of an identical solution with enzyme but lacking substrate and subtracted; a unit of enzyme activity was defined as that amount which caused a density increment of 1.0 in the final diluted sample.

Modified pH 7.5 Ribonuclease assay - The exact procedure was followed as described under (A) of this section with the modification that matched pairs of assay tubes were used, one pair containing 0.3% solution of RNA substrate while the second pair contained an equivalent 0.3% solution of

"core" for each enzyme sample studied.

F. Nitrogen determination.*

The nitrogen concentration of samples was determined by the micro-kjeldahl procedure of Perrin (68).

III. Mouse Pancreas Ribonuclease.

A. Direct extraction from the pancreas.

1. Starting material.

Mouse pancreas was used as a source of enzyme throughout this study. Male mice of the appropriate strain (see under Materials) were quickly killed by placing two fingers of one hand behind the occipital bone and pulling the tail with the other hand to sever the spinal cord. The pancreas was quickly excised and placed on filter paper saturated with physiological saline in a vessel surrounded by crushed ice. During the initial phase of this problem the tissue was homogenized directly in 0.25 N H_2SO_4 or demineralized water. In later studies both 0.2 M phosphate buffer and 0.25 M sucrose were used. Approximately 3 minutes were required for complete homogenization, after which the tissue homogenate was used to prepare a suitable extract or the homogenate was immediately frozen and stored at -20°. An aqueous homogenate of mouse pancreas stored at this temperature maintains its ribonuclease activity undiminished for periods of over a year and is suitable for the preparation of the enzyme. However, in the above scheme, the tissue should not

* The author wishes to thank Dr. J. Van Lancker for the nitrogen determinations on the isolated cell fractions presented in Table VII.

be allowed to remain intact at ice-bath temperatures for periods greater than 15 to 20 minutes or pronounced shifts will occur in the ribonuclease elution pattern. The excised tissue should be homogenized as soon as possible in the appropriate medium and frozen, or an extract prepared and chromatographed immediately.

2. Preparation of an extract.

1. A modified Hirs, Stein, and Moore procedure.

The pooled pancreatic tissue from three or four mice is homogenized in 3.0 ml. of 0.25 N H_2SO_4 for 2 minutes using a Potter-Elvehjem (69) homogenizer equipped with a teflon pestle. The homogenate is stirred at 4° for 10 minutes and centrifuged for an additional 10 minutes in a clinical centrifuge (head No. 213) at 3,000 rpm. The upper layer is decanted including a slight layer of mucoid and fat droplets. By careful addition of 1 N NaOH the pH of the supernatant is brought to between pH 5.5 and 6.0, and the solution is allowed to stand in an ice-bath with occasional stirring, for 10 minutes. The flocculent precipitate that forms is removed by centrifugation as before, and the extract is used for chromatography immediately.

2. A phosphate buffer extraction.

The most convenient procedure for preparing a crude extract of mouse pancreas is to homogenize directly in 0.2 M phosphate buffer at pH 6.47. The homogenate is stirred for 10 minutes at 4° and centrifuged under the same conditions used in the Hirs et al. procedure. 1 to 2 ml. of the thick, viscous supernatant is then chromatographed immediately.

IV. Chromatographic Method.

1. Preparation of resin - The resin used in these experiments was Amberlite IRC - 50 (XE - 64), which is a form of IRC - 50 obtained from the Rohm & Haas Company, Philadelphia. Satisfactory chromatographic results have been obtained equally well with either the finely powdered resin (200 to 400 mesh) or with the coarser product, ranging from 50 to 400 mesh, from which only the "fines" have been removed. In general, the later is preferred for work with crude tissue extracts, since a column of finer particles has a tendency to become clogged at the top.

Before use, the commercial resin must be further purified. For this purpose the resin was suspended in water and allowed to settle for 20 to 30 minutes to remove the fines. This procedure was repeated until the supernatant fluid was clear. The resin was air dried, and treated successively with acetone, water, NaOH, water, HCl, and finally washed with water according to the procedure of Hirs, Moore, and Stein (30,70). The treated resin can then be buffered at the desired pH according to the method of Hirs et al.

2. Preparation of columns - Analytical scale columns are conveniently prepared in chromatography tubes with an inside diameter of 0.9 cm., equipped with a sintered glass plate at the bottom. The wet resin, equilibrated with the appropriate buffer, is suspended in 2 or 3 volumes of buffer and introduced into the tube and allowed to settle by gravity. On completion of a column 30 cm. in length, 200 to 300 ml. of buffer are permitted to pass through the column to pack the resin. At pH 6.47 the

hold-up volume of a column is approximately 0.31 ml./cm.^{3} of resin bed or about 6.5 ml. for a column 0.9 x 30 cm.

3. Operation of columns - Samples to be added to the columns should preferably be of a minimum volume - usually between 1 and 2 ml. total volume. The samples are introduced without disturbance of the column surface by means of a pipette. The sample should be added just as the residual buffer disappears below the resin surface, and the sample washed in with 0.3 to 0.4 ml. of buffer as it in turn disappears below the resin surface. Thereupon, a layer of approximately 5 cm. of buffer is introduced above the column and a solvent reservoir is attached. The most convenient procedure for analytical work is to use a 300 ml. separatory funnel equipped with a constant head air-inlet tube for the reservoir. The funnel is attached by a length of tygon tubing to the column and may, therefore, be used as a leveling bulb to control the rate of flow in the column.

The column is mounted on a fraction collector, and elution allowed to proceed at a rate of 1.5 to 2.0 ml./cm.^{2}/hr. The eluant commonly employed is 0.2 M phosphate buffer at pH 6.47 (thymol saturated).

The IRC - 50 columns may be used repeatedly for tissue extracts if about 20 hold-up volumes of buffer are run through the column between experiments. Usually, it is possible to use a column for only about four chromatograms before the surface becomes clogged. When this occurs, the upper portion of the column may be stirred up and the resuspended resin replaced by fresh, equilibrated resin.

4. Analysis of effluent fractions - Ribonuclease activity and ninhydrin color values were determined on 0.1 ml. aliquots of the effluent as outlined under "Methods". Possible interference of 260 mμ absorbing material eluted with the enzyme was determined by "dead enzyme control" values on the peak fractions. All corrected absorbancy values greater than 0.01 and 0.02 for ribonuclease activity and ninhydrin color, respectively, were considered significant and appear in the "Results" section.

All blanks and standards contained 0.1 ml. of the eluting medium.

V. Cell Fractionation.

1. Homogenization.

The pancreatic tissue from 15 to 20 male grey mice, 2 to 4 months of age, were excised after severing the spinal column. The glandular tissue (ca. 3.0 gm.) was pooled, and homogenized at 0 to 4° in 0.25 M sucrose using a Potter-Elvehjem teflon homogenizer for a two minute period. The final dilution of the homogenate was regularly adjusted to 1:10 (tissue weight: final homogenate volume).

2. Fractionation.

a. Differential centrifugation - The cell fractionation studies were performed using the differential centrifugation procedure of Hogeboom et al. (71). An attempt was made to separate from pancreas homogenates several fractions in addition to the usual nuclear, mitochondrial, microsomal, and supernatant of current fractionation schemes. Out of a number of procedures and schemes tried for this purpose, the following was finally selected.

Table I

Fractionation Scheme for Isolating Nuclear and Cytoplasmic Particulates from Mouse Pancreas Homogenates Prepared in 0.25 M Sucrose Solution

Cell Fraction	Centrifuge	RPM	Time	g/minutes	No. of Washes
			min.		
Nuclear and dense granule fraction	International No. 2	1,500	10	6,000	4
Cytoplasmic a ("Principle zymogen Fraction")	Spinco Model L	5,000	5	8,400	2
Cytoplasmic b	"	5,000	10	16,800	2
Cytoplasmic c	"	13,000	8	90,000	2
Cytoplasmic d	"	13,000	16	180,000	1
Cytoplasmic e	"	25,000	10	411,900	1
Cytoplasmic f	"	40,000	15	1,580,000	1
Cytoplasmic g	"	40,000	30	3,150,000	1
Cytoplasmic h	"	40,000	60	6,300,000	0
Cytoplasmic i	Final Supernatant				

b. Removal of nuclei and intact pancreatic cells - The homogenate is transferred into two 30 ml. plastic test tubes and centrifuged at 1,500 rpm. (600 g.) for 10 minutes in the International centrifuge (horizontal head No. 250). The supernatant is withdrawn with a capillary pipette, and the residue is resuspended by adding 2.0 ml. of 0.25 M sucrose to the total pellet and rehomogenizing for 60 seconds with the pestle and homogenizer used previously. The suspension is recentrifuged for 10 minutes at 1,500 rpm. in the horizontal head. The supernatant is removed and combined with the first supernatant. The residue or "nuclear fraction" is then washed three times by resuspending in 1.0 ml. of 0.25 M sucrose for 15 to 20 seconds with the teflon pestle fitting loosely into the plastic centrifugation tube and recentrifuging as before. The supernatants from the successive washings are combined with the first supernatants to form the cytoplasmic extract. It should be noted that this fraction initially sediments as a well-packed brownish-red pellet which upon resuspension and repeated washing becomes a fluffy red mass increasing in volume several hundred fold. This residue or "nuclear" fraction is made up to a known volume with either 0.25 M sucrose or 0.2 M phosphate buffer and frozen. This fraction contains all of the nuclei present in the homogenate, the residual intact cells, erythrocytes, probably the heavier secretory granules, as well as a number of free mitochondria. Because of a high contamination of this fraction with "dense" secretory granules (see results), this fraction will be defined as the "nuclei and dense granule" fraction, in contrast to the simpler and more classical nomenclature "nuclear fraction".

c. Isolation of the cytoplasmic particles - The cytoplasmic extract remaining after removal of the nuclei and dense granule fraction is transferred to Lusteroid centrifuge tubes and centrifuged for 5 minutes at 5,000 rpm. (1,680 g.) in the Spinco Model L preparative ultracentrifuge using the number 40 head. At this point the sediment is seen to consist of at least three definite layers: a very small bright red pellet on the bottom of the tube, a much larger white opaque layer, and an upper poorly packed layer of tan material. The opalescent supernatant fluid, together with the poorly packed tan material is removed with a capillary pipette and the pellet resuspended in the lusteroid tube with 0.5 to 1.0 ml. of 0.25 M sucrose using a well fitting plastic pestle on a stirring motor. The resuspended pellet was recentrifuged as before and a small amount of poorly sedimenting material is again obtained and removed along with a less opalescent supernatant. This washing procedure is repeated and the resulting clear supernatant combined with the first two supernatants to form a new cytoplasmic extract. The residue or "cytoplasmic pellet No. a" is taken up in a known volume of 0.25 M sucrose or 0.2 M phosphate buffer and frozen.

An identical separatory approach was followed on each of the successive cytoplasmic fractions, with the number of washes as indicated in Table I. Macroscopically the pellets isolated by differential centrifugation appeared opaque and a light tan color in fractions b through e, but changed markedly to a reddish-brown pellet in fraction f, while the following microsomal pellets were red and translucent. A relatively thick, opaque, white pellicle of fat droplets was found on top of the

supernatant after centrifuging down the nuclei and dense granules and the "principle zymogen granule" fractions, and a thinner red pellicle formed on top of the supernatant during the sedimentation of the microsomes.

d. Chromatography of cell fractions - When cell fractionation studies were carried out for the purpose of chromatographic analysis, the individual pellets were resuspended in 1.5 ml. of 0.2 M phosphate buffer at pH 6.47. The buffer-particulate suspensions were subjected to freezing and thawing a minimum of three times to disrupt intact granules. 0.5 ml. was removed, combined with 1.0 ml. of additional buffer and chromatographed immediately. This sample will be designated as the "untreated" phosphate buffer extract. An additional 0.5 ml. aliquot was treated by the procedure of Hirs et al. and chromatographed on the same resin bed after the completion of the "untreated" chromatography. This later sample will be designated as the "Hirs, Moore, and Stein extract".

VI. Incubation Technique.

Animals - Male CBA mice weighing 20 to 25 grams and ranging in age from two to four months of age were used. This age range was found acceptable for general studies, but for individual experiments more uniform results were obtained from mice born within (not more than) two weeks of each other, or preferably litter mates. The mice were either in-bred laboratory animals from our own colony or were obtained from the Department of Anatomy, University of Utah, Salt Lake City, Utah. The animals were maintained on a diet of Rockland Mouse diet and water supplemented with vitamins.

Preparation and Incubation of Mouse Pancreas - The following procedure was found to give good results and was adopted for the preparation of the tissue. The mice were killed by cervical dislocation and a transverse dorsal incision was made in the postlumbar region. The skin was then pulled forward over the head, and a substernal incision was made in the abdominal musculature, exposing the spleen and pancreas. The spleen was carefully raised with a pair of forceps and freed from its thin mesenteric attachment to the pancreas. The pancreas can then be lifted with a minimum of pressure and excised from its duodenal attachment. Care was taken to avoid severing the hepatic artery to prevent contaminating the tissue with blood. The excised gland was placed on a piece of chilled filter paper saturated with physiological saline, and the lobes very carefully freed of connective and adipose tissue. Because it is sufficiently thin and spongy (see text - "Results"), mouse pancreas need not be sliced in order to get adequate oxygenation. Unless otherwise specified, the tissue was immediately weighted on a Torsion balance and placed in conical manometer flasks (6 ml. volume) containing Medium III of Krebs (72) at pH 6.90, precooled to 8°. The center-well of each manometer flask contained a one-inch square of filter paper saturated with 0.1 ml. of 3 N sodium hydroxide. A single, intact pancreas, 100 to 150 mg. of tissue (wet weight), was placed in each flask. In the initial experiments nonincubated weighted pancreas were dried in an oven overnight at approximately 105°, and the ratio of the dry weight to the wet weight (about 0.23) was thus obtained. From this ratio the initial dry weights of the incubated tissues were calculated.

The manometer flasks were kept in a tray of ice-water (8 ± 2°) before they were attached to the manometers. From 15 to 20 minutes elapsed from the start of killing until all the vessels were transferred to the 37° Warburg bath. The tissues were gassed for two minutes with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide, solutions tipped in from the side-arms, and incubated for two hours unless otherwise specified. In all the experiments the gas exchanges were recorded.

Treatment of Tissues and Media after Incubation - Immediately after incubation the vessels were returned to a tray of ice. The tissues were removed from the vessels with forceps and homogenized individually in 3.0 ml. of either demineralized water or 0.25 N sulfuric acid. Portions of the media and homogenate were each diluted with the appropriate buffer. The degree of dilution was determined by the expected enzyme activity. The same dilutions were used in any one series of experiments. These solutions were either immediately assayed or frozen and stored in the refrigerator and assayed on the next day. No change in enzyme activity was observed when either the diluted enzyme solutions or the original samples were stored at -20° for periods up to one year.

Methods of Assay - Amylase was assayed by a slight modification of Somogy's original method (64) and ribonuclease was assayed by the method of Dickman et al. (62). For details of the procedures see under "Methods". The enzyme activities were expressed in relative activity units as mentioned in the experimental section per mg. initial dry weight of tissue. The sum of the medium and tissue enzymatic activity compared to the activity of the medium alone was referred to as the "secreted activity".

Column Chromatography of the Incubated Samples - The chromatographic method used was that developed by Hirs, Moore, and Stein (70) and described under "Methods". Sodium phosphate buffer (0.2 M), pH 6.47, was used, with a column 0.9 x 30 cm.

The incubation media and the pancreas homogenates were pooled from duplicate studies on maximally stimulated tissue (i.e., two manometer vessels containing tissue treated with 1.0 mg. per cent pancreozymin or 5×10^{-7} M pilocarpine), and aliquots taken and treated by the sulfuric acid extraction procedure of Hirs et al. Aliquote of the acid-treated incubation media and tissue homogenates as well as aliquots of the non-treated media samples were chromatographed on IRC - 50 (XE - 64) cation exchange resin. The effluent fractions were assayed for ribonuclease activity and ninhydrin positive material as described above.

RESULTS

I. Properties of Mouse Pancreas Ribonucleases.

A. Stability - The enzyme at different stages of purification was tested for stability to storage under various conditions. There was no apparent loss of activity over periods of one to two years when homogenates or various extracts were (a) lyophilized, (b) frozen and stored at neutral pH or in 0.25 N H_2SO_4, and (c) chromatographed and the eluate frozen and stored at -10°.

B. Effect of pH - The influence of pH on ribonuclease activity in three different buffers may be seen in Figure 1. The optimal pH appears to lie in the region of 7.5. There is a rapid decline in activity above pH 8.0, and the order of magnitude difference in activity at pH 5.0 and 7.5 has proven useful in measuring the homogeneity of several nucleases isolated from mouse pancreas.

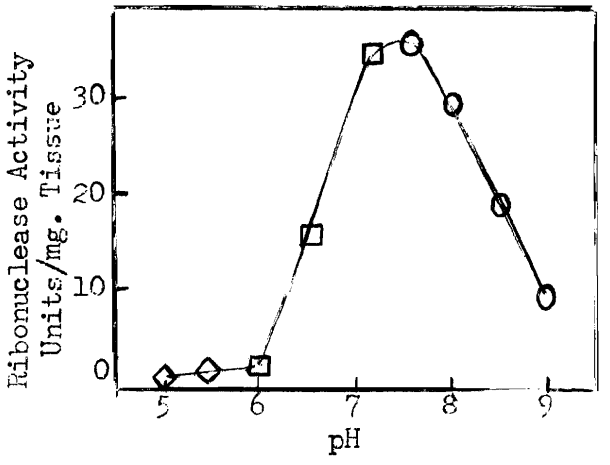


Figure 1. Effect of pH on the rate of hydrolysis of ribonucleic acid by a crude aqueous extract of mouse pancreas. The enzyme assay was carried out as described under "Methods", and the final concentration of buffer was 0.1 M. The symbols are ◇, acetate; □, phosphate; and O, tris.

C. Specificity - Since crystalline beef pancreatic ribonuclease specifically hydrolyzes secondary phosphate esters of pyrimidine nucleoside-3'-phosphates, exhaustive digestion with the enzyme accomplishes only a partial hydrolysis of ribonucleic acid to give pyrimidine mononucleotides and a series of polynucleotides of varying chain length (3,4). These polynucleotides, or "core", contain only one pyrimidine nucleotide residue per molecule, which is in the terminal position and is linked to the remainder of the molecule through its 5'-position. If both the beef and the mouse enzyme act in the same way, then the "core" obtained after exhaustive digestion of ribonucleic acid with the bovine enzyme should not be further hydrolyzed by the mouse enzyme. In fact, as shown in Table II, when 0.4 mg. of "core" was incubated with concen-

Table II

Phosphodiesterase Activity in Mouse Pancreas

For details of the assay procedures see under "Methods". All values in the Table are relative to the activity of a purified spleen phosphodiesterase preparation containing 53 units per mg. protein. Activity of mouse pancreas is stated in absorbancy units per 15.1 mg. of tissue (wet weight).

Enzyme Source	pH 6.5 Assay vs. "Core"	pH 7.5 Assay vs. "Core"	pH 7.5 Assay vs. RNA
	units	units	units
Spleen enzyme	10	2.6	0
Mouse pancreas homogenate	12.6	1.6	904
Crystalline ribonuclease	0	0	1,675

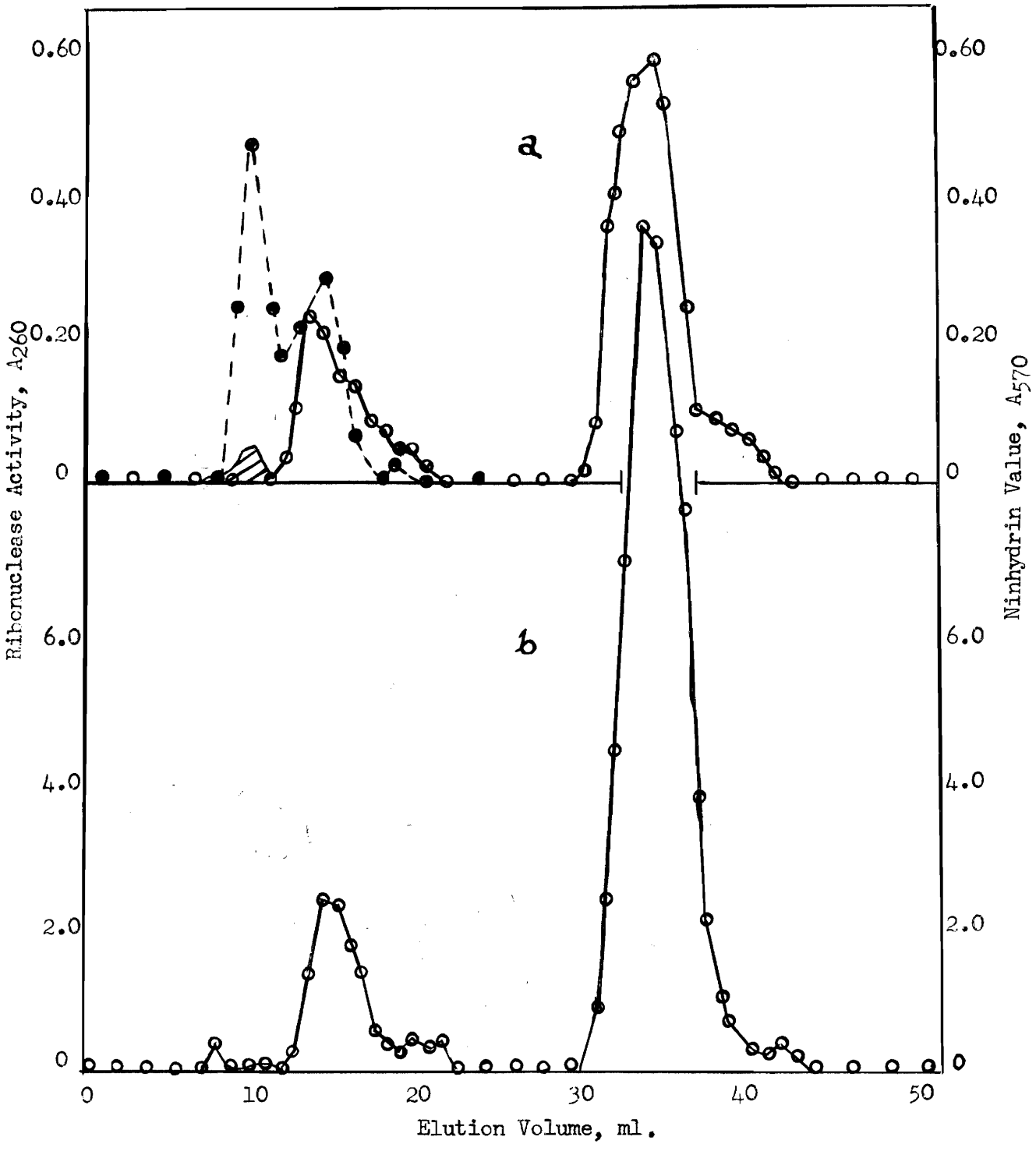
trated extracts of whole mouse pancreas at pH 6.5, appreciable degradation was noted in 30 minutes. However, compared to the total phosphodiesterase activity in the pancreas, more than 99 per cent of the nuclease activity in the homogenate was due to phosphate ester bond hydrolysis at the pH's studied. Thus, the specificity of the mouse pancreas ribonucleases studied in these investigations appears to be similar to that reported by others for beef pancreatic ribonucleases.

D. Chromatography of pancreatic extracts -

1. The Hirs, Moore, and Stein Procedure - In preliminary experiments, the presence of two distinct protein fractions possessing ribonuclease activity in beef pancreas as demonstrated by Hirs et al. (30) made it of interest to determine, if possible, the chromatographic behavior of mouse pancreas ribonuclease. To this end, chromatography was performed on extracts prepared at 4° by the method of Hirs et al. The IRC - 50 columns proved capable of handling the material in the crude tissue extract without apparent distortion of the chromatographic picture. The analytical result is shown in Figure 2. If the effluent volumes were assayed at pH 5.0, the presence of two distinct components possessing ribonuclease activity is apparent. In addition, a third and lesser component is evident at an effluent volume of 10 ml. However, when this component is assayed at pH 5.0 and corrected for "dead enzyme control" values, little ribonuclease activity can be shown, and the peak is apparently largely due to the elution of 260 mμ absorbing material from the column.

Figure 2. Chromatography of a 0.25 N sulfuric acid extract of mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M phosphate buffer, pH 6.47, as the elution agent. a, effluent volumes assayed for ribonuclease activity at pH 5.0, and b, assayed at pH 7.5. For details of the procedures see the text under "Methods". ●, ninhydrin value; O, ribonuclease activity. Shaded area 260 mμ absorbing component.

Note: For the chromatographic elution diagrams of duplicate studies to Figs. 2, 3, 7, 8, 9, 20, and 21, see Appendix.


a
b
0.60
0.40
0.20
0
6.0
4.0
2.0
0
0
10
20
30
40
50
Ribonuclease Activity, A260
Ninhydrin Value, A570
Elution Volume, ml.

Because of the limits of sensitivity of the pH 5.0 assay, comparative values were determined at the optimal pH of the mouse enzyme, pH 7.5. These data are presented in Fig. 2b. The effluents again showed the presence of peaks II and III, and in addition, peak I now becomes a well defined enzymatic entity. It appears, therefore, that there are two and possibly three distinct ribonuclease components in acid-treated mouse pancreas.

To obtain a measure of the protein concentration in the effluent volumes, ninhydrin analyses were performed as previously described under "Methods". A single ninhydrin positive component was eluted at 10 ml. with ribonuclease "I". The position of this peak on the effluent curve, however, was consistently displaced from the enzyme peak, and it seems likely that this component is a contaminant, rather than protein material attributable to the enzyme. The other major ninhydrin positive component moves slightly slower and emerges at 16 effluent ml., closely coicident with ribonuclease "II". This peak is markedly asymmetric, with the ratio of ninhydrin color value to enzymatic activity on the first part of the peak much greater than that on the remainder. Although this asymmetry might result from inhomogeneity, and it has in fact been possible to directly demonstrate the existence of another component in this peak, based on dialysis data to be discussed later, it would appear that much of this ninhydrin positive material can also be considered as a contaminant.

Since the material in peak II, and in many respects that in peak I, is chromatographically indistinguishable from the material designated as

peaks A and B respectively by Hirs, Moore, and Stein in beef pancreas, it became important to determine whether component III might arise from I or II as a result of proteolytic activity. To this end, analyses were carried out on the various extraction steps of the Hirs et al. procedure. The results of this study presented in Table III indicate that while an aqueous homogenate of mouse pancreas contains a high level or proteolytic activity, when freshly excised mouse pancreas is immediately homogenized in 0.25 N H_2SO_4, all measurable proteolytic activity was precipitated. In addition, chromatographic analyses were carried out on an aqueous homogenate which was allowed to stand 6 hours at room temperature before being treated by the Hirs et al. procedure and chromatographed. Although the total ninhydrin value for the sample increased markedly, the enzymatic elution curve was very similar to that shown in Fig. 2a.

2. Phosphate buffer extraction procedure - To determine to what extent the three components in mouse pancreas might be derived from the "native" cellular enzyme(s) by the action of dilute sulfuric acid, an alternate method of extraction was devised by homogenizing the freshly excised tissue directly in the chromatographic eluting agent, the 0.2 M phosphate buffer. The chromatograms, as shown in Figure 3, again show the inhomogeneity revealed by the curves in Figure 2a, but, with the possible exception of peak II, display little similarity to the sulfuric acid extracts. By far the largest part of the eluted activity was present in a broad zone extending from 25 to 45 ml. This single component(s) accounted, within experimental error, for 70 per cent of

Table III

Proteolytic Activity in Mouse Pancreas

For details of the assay procedure see under "Methods". The following samples were used: sample A, a mouse pancreas homogenate frozen and stored for 6 months at -10°, 140 mg. of tissue homogenized in 3.0 ml. of demineralized water. 0.1 ml. used in assay; sample B, a fresh mouse pancreas homogenate, 136 mg./3 ml. The aqueous homogenate was treated by the method of Hirs et al. 0.1 ml. of each sample used in assay. A/280 corrected for DEC.

Enzyme Source	A/280 mμ	Tryptic Activity γ/100 mg. Pancreas
10γ Crystalline trypsin	0.060	....
Mouse pancreas (sample A)	0.109	404
Mouse pancreas (sample B)		
a. Aqueous homogenate	0.086	322
b. 0.25 N sulfuric acid extract of a.	0.170	630
c. Final pH 5.8 supernatant	-0.004	<5*

* Assay sensitive to less than 5 gamma of crystalline trypsin.

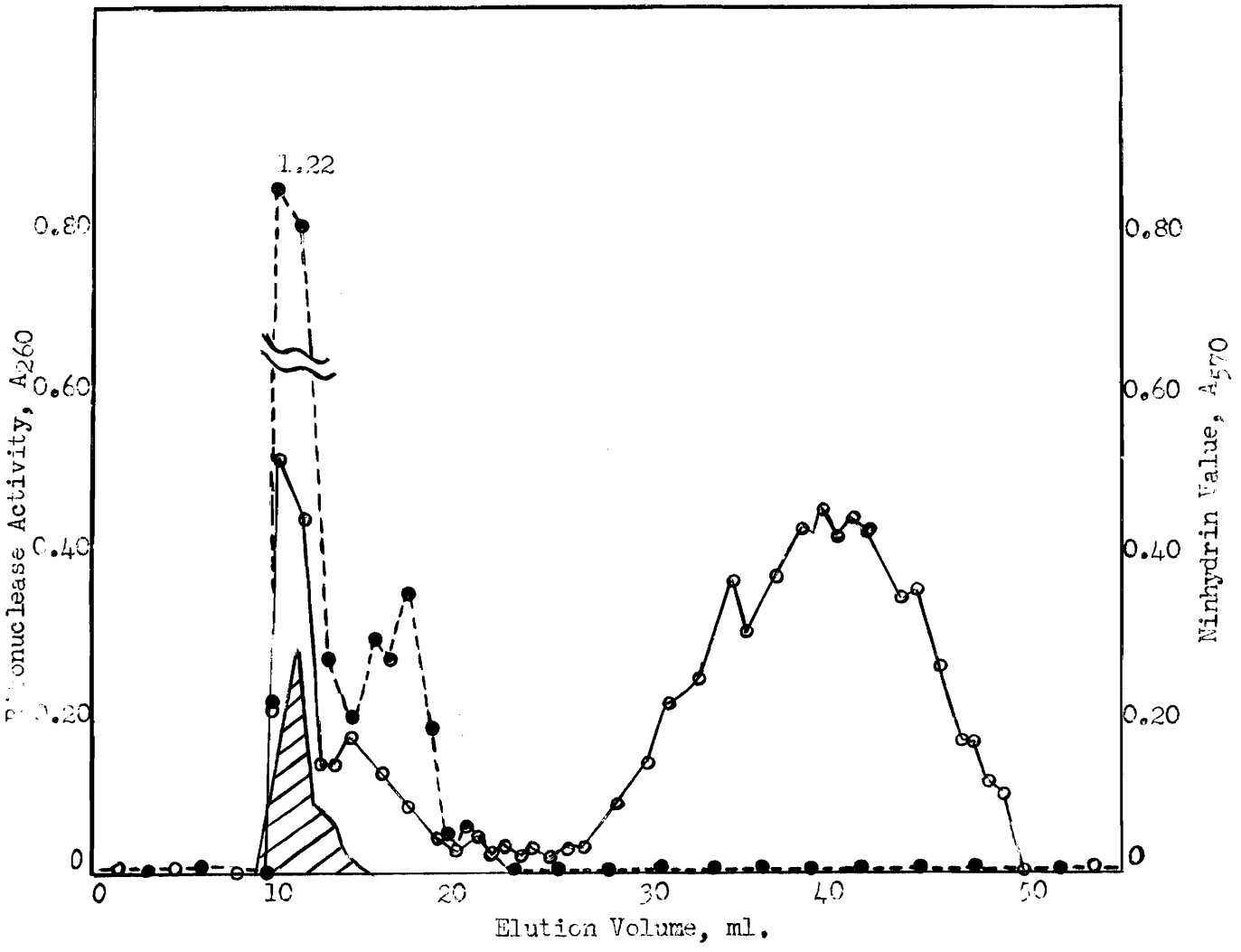


Figure 3. Chromatography of a sodium phosphate buffer extract (0.2 M, pH 6.47) of mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer, pH 6.47, as the elution agent. Effluent volumes assayed for ribonuclease activity at pH 5.0. For details of the procedures see text under "Methods". ●, ninhydrin value; 0, ribonuclease activity measured at pH 5.0. Shaded area, 260 mμ absorbing component.

the activity applied to the column. In addition, the presence of two lesser enzymatically active components is evident in Figure 4, and have the same elution volumes, 10 and 16 ml., as peaks I and II, respectively, from the sulfuric acid extracts. For simplification and clarity these two extraction methods will be simply designated as acid-treated (Hirs et al. procedure) and as non-acid-treated (sodium phosphate buffer extraction).

The presence of other contaminating components are worthy of mention. The first of these is the appearance of 260 mμ absorbing material at elution volumes 11 and 12, coinciding with peak I from the phosphate extract. A comparison of the quantitative elution of this material from the acid-treated (Fig. 2a) and the non-acid-treated (Fig. 3) tissue extracts indicates that sulfuric acid treatment removes 80 to 90 per cent of the A_{260} absorbing material in an aqueous pancreas extract. As mentioned above, the second major contaminant emerges as two well-defined ninhydrin positive components with the I and II components of ribonuclease activity. It appears that the ninhydrin positive material from the non-acid-treated tissue extracts is chromatographically indistinguishable from the same material eluted from the sulfuric acid extracts.

3. Homogeneity of the individual Ribonuclease components - It is clear that the two tissue preparations differ in heterogeneity when examined chromatographically. They both have, however, three major peaks which will be designated ribonuclease "I", "II", and "III", respectively. It is important to note, moreover, that the particular chromatographic procedures utilized in the foregoing studies cannot, in them-

selves, provide evidence of the homogeneity of the individual peaks. Thus, in order to more carefully evaluate the enzymatic systems under investigation, additional criteria must be devised to determine the actual degree of heterogeneity of the pancreatic enzymes. To this end, four criteria will be presented. These are: a) rechromatography; b) gradient pH elution; c) Ribonuclease-pH activity ratios; and d) subcellular localization.

a. Rechromatography - When sulfuric acid extracts were chromatographed on columns of IRC - 50 (Fig. 2), definite evidence of inhomogeneity was observed in each of the two main peaks. One obvious indication of inhomogeneity was the apparent asymmetry of these peaks. It could not be decided whether the asymmetry of the main peak, for example (see Fig. 1a in the Appendix), was a result of anomalous chromatographic behavior, or whether it could be attributed to the presence of a mixture of enzymatic species. When material from peak III in Figure 2a was rechromatographed under the same conditions, a single asymmetric peak in the same position (Fig. 4) was again obtained, demonstrating that passage through the column had not affected the protein detectably.

b. Gradient pH elution - Since the charge on the protein depends upon the pH, the position of the peak on the effluent curve should be altered by varying the pH of the eluting buffer. Thus, chromatography of the enzymes over a suitable gradient pH range should provide further evidence as to the nature of the protein species involved. In these studies (Fig.5a & b) gradient elution was affected by a buffer of

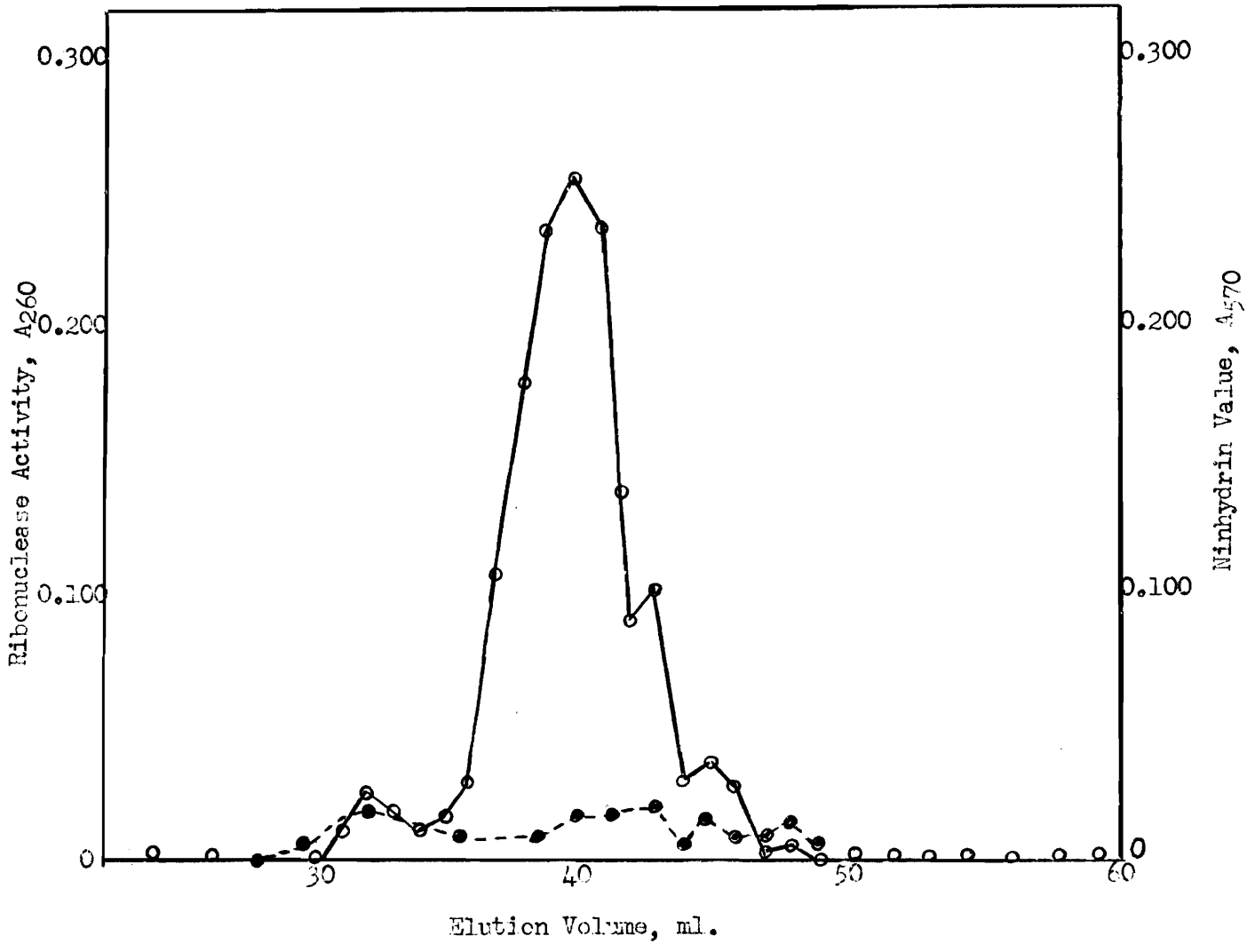
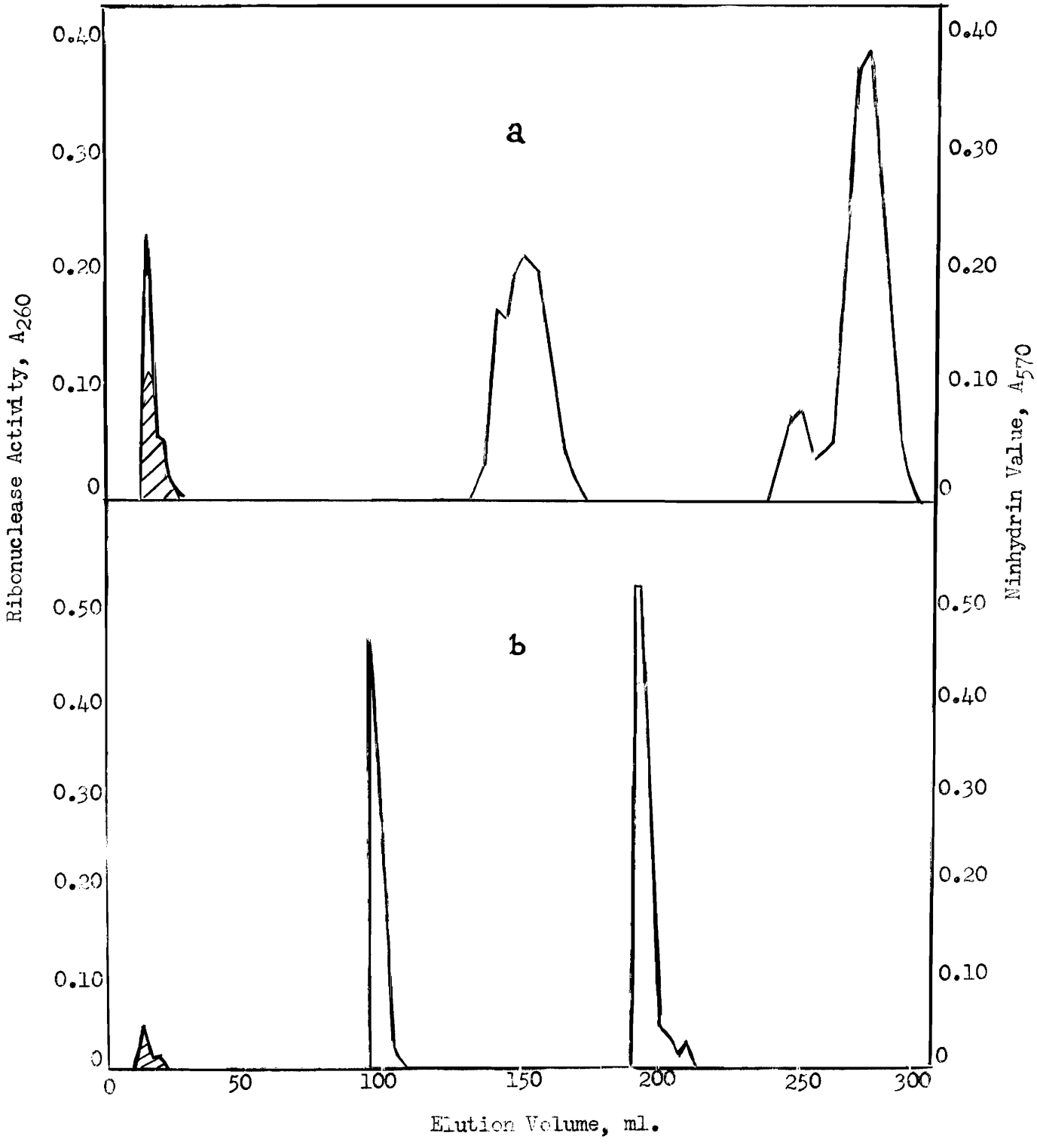


Figure 4. Rechromatography of peak III from a sulfuric acid extract of mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer, pH 6.47, as eluent. The ribonuclease I and II peaks were not present and thus this region of the effluent curve is not shown. ●, ninhydrin value; 0, ribonuclease activity measured at pH 5.0. Recovery of ribonuclease activity, 98 per cent.


a
b
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Elution Volume, ml.
0.40
0.30
0.20
0.10
0
0.50
0.40
0.30
0.20
0.10
0
0
50
100
150
200
250
300

constant ionic strength and increasing pH. As would be expected, there is a considerable retardation volume before a change in pH is reflected in the effluent. When chromatographed under these conditions the mouse pancreas ribonucleases appeared as two major asymmetric peaks from both the acid-treated and non-treated tissue extracts. For comparison in each of these studies, aliquots of the extracts were analyzed on control IRC - 50 columns at pH 6.47, and elution patterns similar to those in Figures 2a and 3 were found. With pH gradient elution chromatography the peaks obtained from the sulfuric acid extracts were sharp, while those from the untreated phosphate buffer extracts were broader and emerged more slowly from the column than the former. In addition, with gradient elution, a third non-enzymatic component of 260 mμ absorbing material appears near the column volume, indicating that the charge on the material in question is relatively stable to small changes in pH. In general, this material appears to be similar to the 260 mμ absorbing material eluted at the same effluent volume coincident with ribonuclease I.

c. Ribonuclease-pH activity ratios - In the interpretation of effluent curves, chromatographic data alone cannot provide decisive data concerning the nature of the protein species involved. It is thus equally important to characterize the material in the various peaks from the chromatograms by several independent methods. A second important point of reference in the present studies, therefore, is enzymatic activity. However, of the three enzyme parameters involved in these studies, stability, specificity, and pH-activity, only the latter presents any strong evidence for the uniqueness of the individual

Table IV

Ribonuclease pH-Activity Ratios for Mouse Pancreas Homogenates and Column Effluent Fractions

The individual effluent volumes from peaks I, II, and III of several Hirs, Stein, and Moore chromatographic studies were assayed for ribonuclease activity at pH 5.0 and 7.5. The figures in brackets in column three represent the range of the individual eluates comprising the peak. The homogenate is the final Hirs *et al.* supernatant adjusted to pH 6.0. For details of the procedures see under "Methods".

Fraction	Number of Analyses	Activity at pH 7.5 / Activity at pH 5.0
Homogenate	3	15.3 ± 0.1
Peak I	7	* (20-70)
Peak II	10	12. (9.5-16.7)
Peak III	13	22. (21.-25.)
Crystalline beef RNase	3	26.3 ± 1.8

* Large variation in 7.5: 5.0 activity ratios due to low activity of peak I at pH 5.0 (see text - Fig. 3).

peaks. The results in Figures 2a and b clearly indicate that there is at least a two-fold difference in the pH 7.5:5.0 enzyme activity ratios of ribonuclease II and III from the sulfuric acid extracts. To explore this variable further, analyses were performed on the peak tubes from a number of chromatograms (Table IV). These data indicate that although the pH-activity ratios are relatively constant for ribonuclease III, the pH-activity ratios for ribonuclease II were markedly asymmetric, with the ratio on the first part of the peak only somewhat more than half (9.5) that on the remainder (16.0).

d. Sub-cellular localization - On the basis of the results described above, the evident inhomogeneity revealed by the chromatographic elution curves made it of interest to determine, if possible, the localization (and thus fractionation) of individual ribonuclease components in specific sub-cellular structures. As the results reported in this thesis will show, however, this study proved to have extensive implications, and will be presented in a section dealing exclusively with the intra-cellular distribution of mouse pancreas ribonuclease.

E. Activation and inhibition: Recovery of ribonuclease activity and ninhydrin positive material from mouse pancreas homogenates - It is clear that before a concise picture of a complex cellular enzyme can be reconstructed, the quantitative relationships of the recoveries of enzymatic activity during the various preparative and analytical procedures must be carefully considered. With this in mind, the recovery of both the total protein (as ninhydrin positive material) and ribonuclease activity from the steps in the procedure of Hirs et al. as well as

column eluate recoveries are presented in Tables V and VI. Somewhat more activity was consistently found in the pH 5.8 supernatant than in the original homogenate. In view of the fact that this solution before centrifugation did not exhibit this increase, it is possible therefore, that an inhibitor may become insoluble at pH's of 5.5 and 6.0 and be removed in the centrifugation, whereas it would still be present when the entire solution was assayed. Furthermore, a comparison of the curves obtained with the Hirs et al. extract (Fig. 2a) and those obtained with the phosphate buffer extract (Fig. 3) indicate that when the individual eluates are totaled, only 70 per cent of the activity placed on the Hirs et al. column was recovered compared to a quantitative recovery for the untreated extract column. On the other hand, if the eluate from the Hirs et al. column was collected as a single fraction, its total activity equalled that of the original extract. Thus these data also suggest the presence of a ribonuclease inhibitor in mouse pancreas which may be concentrated in certain eluates and thus be more effective than when distributed through-out the entire volume. Direct evidence of the actual location of this inhibitor, in fact, is suggested by the pH gradient elution curve (Fig. 5). When the individual eluent activities from this column were totalled, 100 per cent of the activity placed on the column was recovered, with the necessary increased activity appearing in the peak theoretically corresponding to ribonuclease II. The isolation of this inhibitor in mouse pancreas extracts or column eluates, however, has not yet been obtained.

Table V

Recovery of Ribonuclease Activity and Ninhydrin Positive Material from Mouse Pancreas Homogenates and the Various Steps of the Hirs et al. Tissue Extraction Procedure

Data are average values for duplicate studies. For details see under "Methods".

Fractionation Step	Ribonuclease Activity pH 5.0	Ninhydrin-positive Material
	percent	percent
Aqueous pancreas homogenate	100	
Sulfuric acid homogenate	97	100
Sulfuric acid supernatant	91	....
pH 5.8 supernatant	121	37
Column eluates (summation)	93 (70)*	1-2
Column eluates (single volume)	103**	....

* Recovery calculated from summation of individual eluates as per cent of total activity placed on column. See Table VI.

** In these experiments the eluate was collected in one flask and its total ribonuclease activity calculated as per cent of the total activity placed on the column.

Table VI

Chromatographic Distribution of Mouse Pancreas Ribonucleases

Ribonuclease activity was assayed by the standard procedure at pH 5.0. The recovery was calculated from the summation of individual eluates as per cent of the total activity placed on the column. The values for the pH 6.47 elution chromatography of the Hirs et al. extract represent average values for five studies. All other values are averages for duplicate studies. Peaks I, II, and III from the two pH gradient elution studies are so designated by the order of their elution, and do not necessarily appear at the same effluent volume as the corresponding peaks from the control pH 6.47 elution chromatography (see Fig. 4).

Chromatographic Study	Per Cent of Total Eluted RNase Activity			% Recovery
	Peak I	Peak II	Peak III	
Hirs et al. extract				
pH 6.47 elution	2.5	28	68	66-72*
pH gradient elution	2	45	54	102
Phosphate buffer extract				
pH 6.47 elution	11	10	79	101
pH gradient elution	2	27	71	114

* Range for five chromatographic studies.

The data of Table V also show that about 40 per cent of the ninhydrin positive material was removed in the preparation of the pH 5.8 extract and that only about 1 per cent was eluted from the column. As previously mentioned, the material which was eluted with ribonuclease I and II might represent the enzyme protein itself or indicate extraneous substances. This later possibility is more likely since the ninhydrin peaks do not coincide with the ribonuclease activity peaks and, as indicated in Figure 16, much of this material could be removed by dialysis without affecting the position or heights of the ribonuclease peaks.

II. Intra-Cellular Localization of Mouse Pancreas Ribonucleases.

The apparent inhomogeneity revealed by the chromatographic elution curves in the preceding section made it of interest to determine, if possible, the chromatographic behavior of "native" ribonuclease as it exists in specific intra-cellular structures in the pancreas. It was anticipated that perhaps these enzymes would be associated with certain cellular components and thus cell fractionation techniques coupled with analytical chromatographic methods would provide further information on the ribonucleases of mouse pancreas.

A. Distribution of ribonuclease activity in mouse pancreas cell fractions - The ribonuclease activities of mouse pancreas and the cell fractions isolated from it are presented in Table VII. A more illustrative survey of the results recorded in Table VII is provided by the graphs of Figures 6a and b, and is a composite of a series of experiments assayed for ribonuclease activity at pH 5.0. Figure 6b was constructed by plotting

Table VII

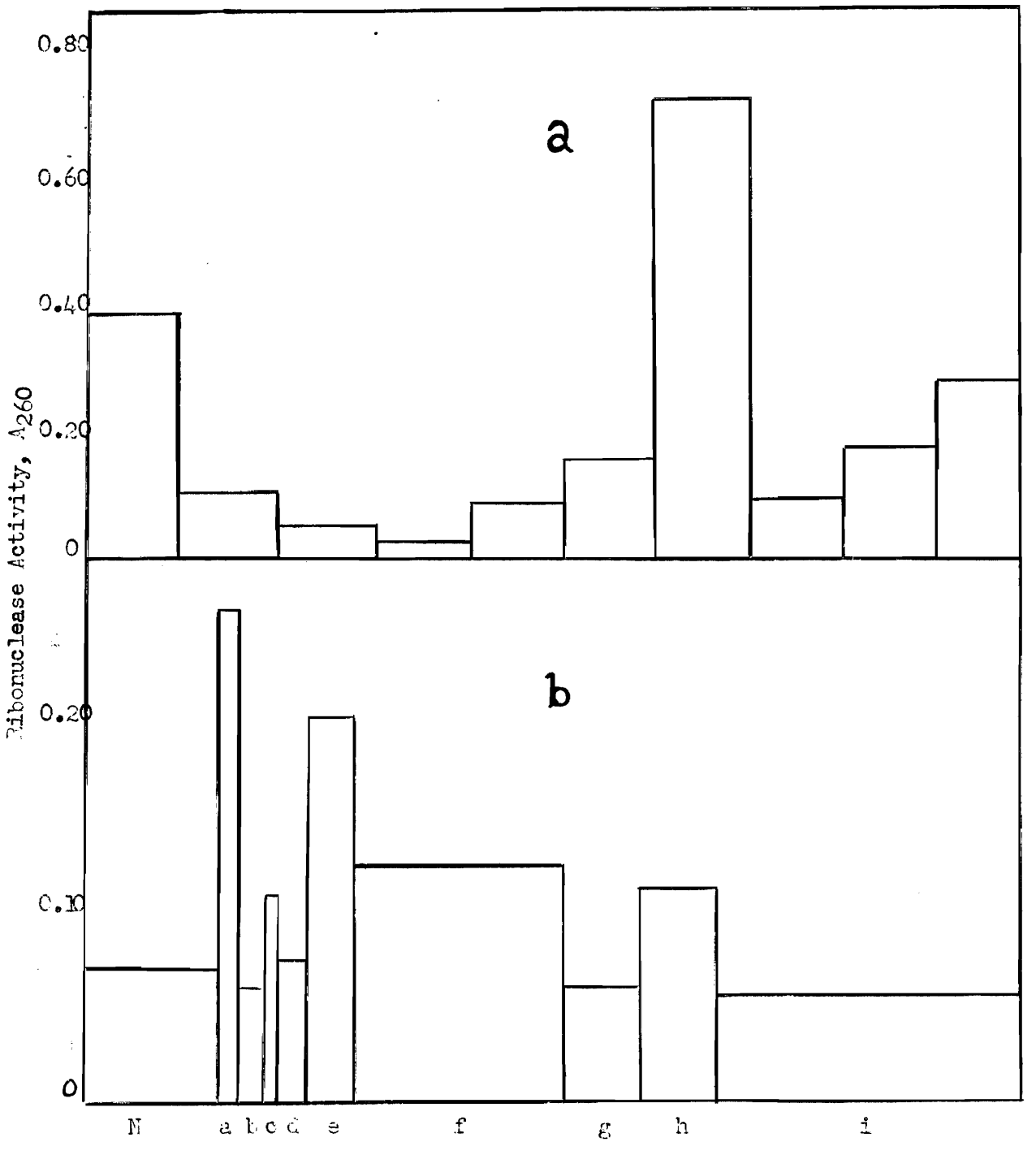
Distribution of Ribonuclease Activity in Cell Fractions Obtained from Mouse Homogenates Prepared in 0.25 M Sucrose

The values reported in this table represent composite data from four fractionation studies. Individual pellets taken up in 1.5 ml. of 0.2 M sodium phosphate buffer, pH 6.47, and aliquots diluted in the appropriate assay buffer. Recovery calculated as per cent of the total activity in the isolated cell fractions. Ribonuclease activity determined by the standard pH 5.0 and 7.5 assay. See Table I for fraction nomenclature

Pancreas Fraction	Total Nitrogen	Ribonuclease Activity	
		pH 5.0 Assay	pH 7.5 Assay
	mg.	per cent	per cent
Nuclear	4.0	14.5	16.7
a	0.5	11.1	6.2
b	0.9	3.0	2.7
c	0.5	1.9	1.8
d	1.0	4.2	3.6
e	1.3	10.1	5.8
f	5.8	34.6	35.5
g	2.3	5.5	4.6
h	2.0	3.5	8.2
Supernatant	9	11.5	14.1
Recovery*	94%	70%	67%

* Recovery calculated from summation of the individual fractions as per cent of the total activity in the original homogenate.

Figure 6. Intracellular distribution patterns of mouse pancreas ribonucleases. a, relative ribonuclease activity (per cent of the total activity) in the fractions in the order in which they were isolated, i.e., from left to right; Nuclei, a, b, c, d, e, f, g, h, and i (see Table I). b, mean relative specific activity of fractions; fractions are represented by their relative nitrogen content, in the order in which they were isolated. For description of patterns see text.


0.80
0.60
0.40
0.20
0
a
Ribonuclease Activity, A260
0.20
0.10
0
b
N
a
b
c
d
e
f
g
h
i

the mean relative specific activity of the fraction against the mean relative nitrogen content. The area of each block is thus proportional to the percentage of the activity recovered in the corresponding fraction, and its height to the degree of purification achieved over the homogenate. As the results show, the major portion of the enzyme activity (~ 70 per cent) appeared to be associated with three specific fractions in the pancreas. Of the three, about 20 per cent of the total ribonuclease activity in the tissue was recovered in the nuclear fraction and about 15 per cent in the final supernatant. The activity of the nuclear fraction must apparently be accounted for mainly by contaminating zymogen granules, although this point should be carefully established by analyses of a purified nuclei fraction. At this point it is not known whether the activity in the final supernatant could be considered a true representation of the situation in situ, or whether it was due to leakage from granules during tissue homogenization and differential centrifugation. The activity in the supernatant, or of its subfractions, will be considered in detail in connection with the chromatographic elution studies. The most extensive concentration (~ 36%) of ribonuclease activity, however, occurred in a fraction identified as a cytoplasmic microsomal fraction (f). This fact suggests that the extra-zymogen granular ribonuclease has a single intra-cellular location with a markedly unequal distribution in the microsomal sub-fractions. However, the ease with which this enzyme may be chromatographically eluted from the particles (Table X) suggests that the ribonuclease is not truly microsomal but belongs to the non-particulate portion of the cell, and is secondarily adsorbed on the microsomes. This explanation is, however, not supported by

a comparison of the chromatographic elution curves from the soluble supernatant enzyme and the microsomal enzyme. In addition, it has been shown by Van Lancker (73), in collaborative studies with these cell fractions, that certain other enzymes, i.e., amylase, acid phosphatase, and deoxyribonuclease, also are localized in this specific fraction of the pancreas, adding further support for the uniqueness of this structural cell component.

In these studies, it was found that the ribonuclease of the large granules and microsomal particles were readily released by treatment with 0.25 N sulfuric acid or 0.2 M phosphate buffer at pH 6.47 with variable recoveries, depending upon the fraction and the method of treatment (Table VIII). Repeated freezing and thawing were essentially without effect on the total recovery of ribonuclease activity from the particulates.

In all cases, the recoveries of ribonuclease activity in the cell fractions ranged between 65 and 75 per cent of the activity of the total homogenate. The excellent recoveries of nitrogen and the more robust enzymes such as acid phosphatase show that no gross errors are introduced by the fractionation procedure itself. This poor recovery of ribonuclease in the pancreas fractions apparently involves some subtle interaction between the fractions, because when they were recombined in the same proportions which existed in the original homogenates, full activity was obtained.

B. Action of 0.25 N sulfuric acid on RNases and cell fractions - It appears that no careful study has been made of the effect of acid on ribonuclease activity of cell fractions or of crude or purified preparations. Thus, since acid is used in the preparation of crystalline beef pancreatic

ribonuclease and in the purification procedure for the mouse pancreatic ribonucleases in the initial studies of this problem, its effect on the ribonuclease activity of various cell constituents and tissue preparations is of interest.

In a typical experiment, illustrated in Table VIII, mouse pancreas homogenates and the ribonuclease rich cell fractions were treated with 0.25 N sulfuric acid in the cold by the procedure of Hirs et al. After adjusting to pH 6.0, the solutions were assayed at pH 5.0. Controls were treated with equal amounts of 0.2 M phosphate buffer at pH 6.47. No significant change was noted in the activity of the acid-treated whole-tissue homogenate compared to an aqueous or phosphate buffer homogenate. Since there was little difference between the water-treated or the phosphate buffer-treated homogenates, results of the former have been omitted. Acid treatment of the isolated cell fractions, however, causes a large loss in the ribonuclease activity of the nuclear fraction but this is almost completely compensated for by increases in the activities of the zymogen, microsomal, and supernatant fractions. The largest part of this increase arises from the acid treatment of the supernatant fraction. In addition, the specific activities of the fractions are greatly increased, in every instance, by acid treatment which, in addition to apparently releasing, in some cases, additional ribonuclease activity, also precipitates a large percentage of the protein of the fraction (see Table VIII). Thus, this one-step procedure results in an increase of 5 to 50 times in the specific activity of the ribonucleases of mouse pancreas cellular fractions.

Table VIII

Effect of 0.25 N Sulfuric Acid on Ribonuclease Activity in Mouse Pancreas Extracts and Isolated Cell Fractions

The pellets from the isolated cell fractions were suspended in 1.5 ml. of 0.2 M phosphate buffer, pH 6.47, and a 0.5 ml. aliquot treated by the procedure of Hirs et al. Ribonuclease activity was determined on aliquots of each sample by the standard pH 5.0 assay. 1.0 ml. of the pancreas extract (homogenate) and final supernatant were added to an equal volume of 0.2 M phosphate buffer and 0.5 ml. removed and treated as above. Ninhydrin positive material determined as described under "Methods". For fraction nomenclature see Table I. Fraction and homogenate activity expressed in relative absorbancy units.

Preparation	Ribonuclease Activity		% RNase Recovery	% Recovery Ninhydrin-Positive Material
	Phosphate Extract	Acid Extract		
	absorbancy units	absorbancy units		
Homogenate	54	52	97	37
Nuclear fraction	25.6	7.3	28	34
"Principle zymogen fraction"	23.8	27.4	115	41
"Cytoplasmic f" microsomal fraction	43.0	55.6	132	46
Final supernatant	5.0	22.8	455	81

C. Chromatographic analysis of ribonuclease rich cell fractions - The chromatographic investigations of ribonuclease activity in whole tissue extracts have been paralleled by studies on the distribution of the various ribonuclease components in sub-cellular fractions. The preliminary whole-tissue studies indicated that at least three major ribonuclease components are present in both the Hirs et al. extract and the phosphate buffer extract. However, as noted previously, the elution patterns from these two extraction procedures are not strictly comparable and peak III from the Hirs et al. extract, for example, should not be considered as the same molecular species as peak III of the phosphate extract. Thus, in this regard, the enzymes in question will be treated as at least six distinct components and will be designated as peaks I, II, and III, treated and non-treated, respectively.

When the homogenate was fractionated and extracts made of the intracellular fractions, these six peaks were not universally present in all fractions. The data on their distribution are given in Table IX. Measurements for peak I of the treated or Hirs et al. extract were not considered reliable enough for tabulation, since with the pH 5.0 assay it is difficult to determine the ribonuclease activity due to interference by the 260 mμ absorbing material eluted in the same position. The data clearly show that peak I from the non-treated fraction extracts is located exclusively in the "soluble" portion of the cell. In contrast, however, the remaining peaks are present in varying amounts in all cell fractions, with the possible exception of individual components comprising peak III (to be discussed in a later section).

Table IX

Ribonuclease Components Isolated by Column Chromatography from Pancreas Homogenates and Cell Fractions

Details of the procedures are given in the experimental section. Activity per peak was calculated from a summation of the individual eluates of a given peak as per cent of the total activity eluted from the column. Ribonuclease activity was determined by the standard pH 5.0 assay. Data are average values for duplicate studies. Phosphate extract: extraction procedure using 0.2 M sodium phosphate buffer, pH 6.47; acid extract: Hirs et al. extraction procedure.

Preparation	Ribonuclease Activity Eluted from Column				
	Peak I	Peak II		Peak III	
	Phosphate Extract	Phosphate Extract	Acid Extract	Phosphate Extract	Acid Extract
	per cent	per cent		per cent	
Homogenate	11	10	27	79	70
Nuclear and dense granule	0	21	50	79	50
"Principle zymogen fraction"	0	21	41	79	59
"Cytoplasmic f" microsomal fraction	0	10	21	90	79
"Cytoplasmic g" and "h" microsomal fractions	24	0	...	76	...
Final superantant					
Sample a	13	32	56	56	44
Sample b	70	22	55	8	45
Sample c	17	72	...	11	...
Sample d	31	28	...	41	...

According to their intra-cellular localization, the chromatographic elution patterns can be divided into four groups, which possibly correspond to at least three distinct classes of cytoplasmic granules.

Nuclear and dense granule fraction - The chromatographic elution pattern for a phosphate buffer extract and a Hirs et al. extract of a mouse pancreas nuclear fraction is shown in Figure 7. The chromatographed nuclear extracts demonstrate several important differences from whole-tissue studies: namely, (a) the major component (peak III) of the whole-tissue phosphate buffer extract (text - Fig. 3) came out about 10 ml. earlier with a considerably broader peak compared to the single sharp peak present in the nuclear fraction; (b) in these experiments there was no evidence of peak I in the non-treated extracts and, in addition, there was only a minor front-running 260 mμ absorbing component; (c) peak II shows evidence of a second and possibly a third component immediately preceding and following the major peak at about 18 ml.; (d) after treatment with 0.25 N sulfuric acid, several pronounced changes appear in the chromatographic elution pattern. The major peak, III, now appears as a rather low, broad, indistinct zone in the same elution position as peak III from the non-treated extract. This loss of peak III activity, however, appears to occur on acid treatment of the nuclear pellet, and is not directly concerned with the chromatographic treatment of the extracts (see Table VIII). The fact that no significant over-all loss in activity occurred on chromatography is shown by the results presented in Table X. In this case the difference between the 85 per cent recoveries for the Hirs et al. extracts and the 106 per cent recoveries for the phosphate buffer extracts are not considered

Figure 7. Chromatography of extracts of the nuclear fraction on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a sodium phosphate buffer extract of the nuclear fraction; b, curve obtained from a Hirs et al. extract of the same cell fraction. ●, ninhydrin value; O, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.

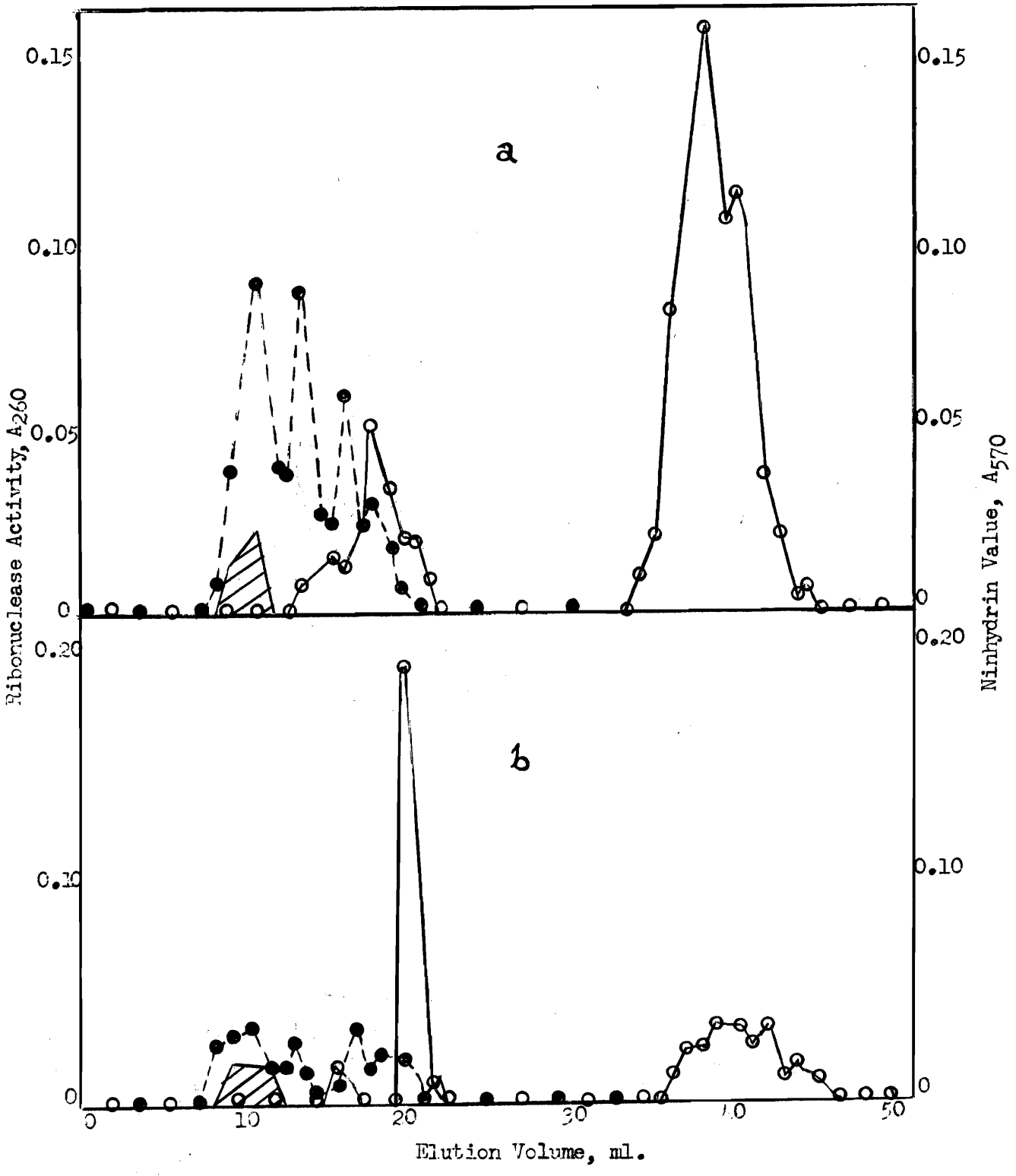

a
b
0.15
0.10
0.05
0
0.20
0.10
0
10
20
30
40
50
Ribonuclease Activity, A260
Ninhydrin Value, A570
Elution Volume, ml.

Table X

Recovery of Ribonuclease Activity in the Column Effluent Fractions from Total Homogenates and Cell Fractions

For details of the extraction and chromatographic procedures see the experimental section. Recovery calculated from summation of individual eluates as per cent of the total activity placed on the column. The data represent average values for duplicate fractionation studies. Ribonuclease activity determined by the standard pH 5.0 assay. For fraction nomenclature see Table I.

Preparation	Recovery of RNase Activity	
	Hirs et al. Extract	Phosphate Buffer Extract
	per cent	per cent
Homogenate	67	102
Nuclear and dense granule fraction	85	106
"Principle zymogen granule" fraction	145	99
"Cytoplasmic f" microsomal fraction	96	143
"Cytoplasmic g" and "h" microsomal fraction	...	220
Final supernatant	100	196-463*

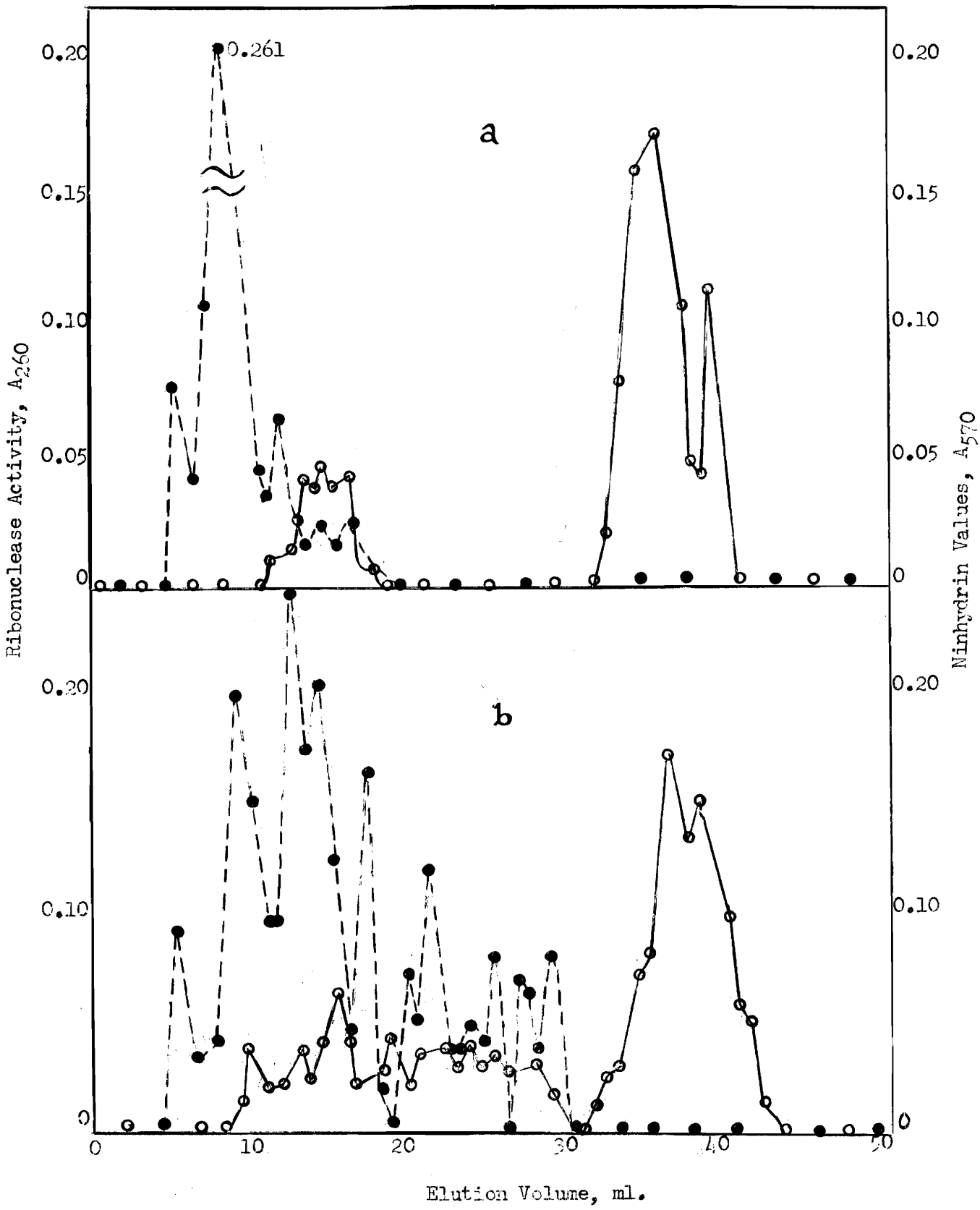
* range for the final supernatants from the duplicate fractionation studies.

significant in respect to the total shift of ribonuclease activity from peak III to peak II as shown in Table IX. This does not eliminate, however, the possibility that one component may be inactivated while the second was activated.

Zymogen granule fraction - The ribonuclease components separated by column chromatography from the nuclear fraction might be explained by contamination with zymogen granules either free or included in cell fragments. Comparative evidence, however, indicates the existence of greater complexities with respect to the so-called "principle zymogen granule fraction" and the nuclear fraction. At first glance, the chromatographic elution pattern for a phosphate buffer extract of the zymogen fraction (Fig. 8b), appears to be identical with the corresponding nuclear extract. On treatment by the Hirs et al. procedure, however, the major peak(III) gave no graphical indication of destruction or conversion, although from the data in Table IX, there appears to be a 15 per cent conversion to material again eluting in the region of peak II. It follows from these data therefore, either, 1) two distinct types of zymogen granules are present in the cell and can be centrifugally separated by means of a difference in density and surface area, or that, 2) the nucleus contains specific ribonuclease components that are distinct from those in the cytoplasm. Evidence for the former possibility will be presented in the section dealing with physiological changes in the pancreas.

Microsomal fraction - The third group of ribonucleases, which comprise the microsomal enzymes, appears to be concentrated to a large extent in a single fraction and may occur to a lesser extent in the other

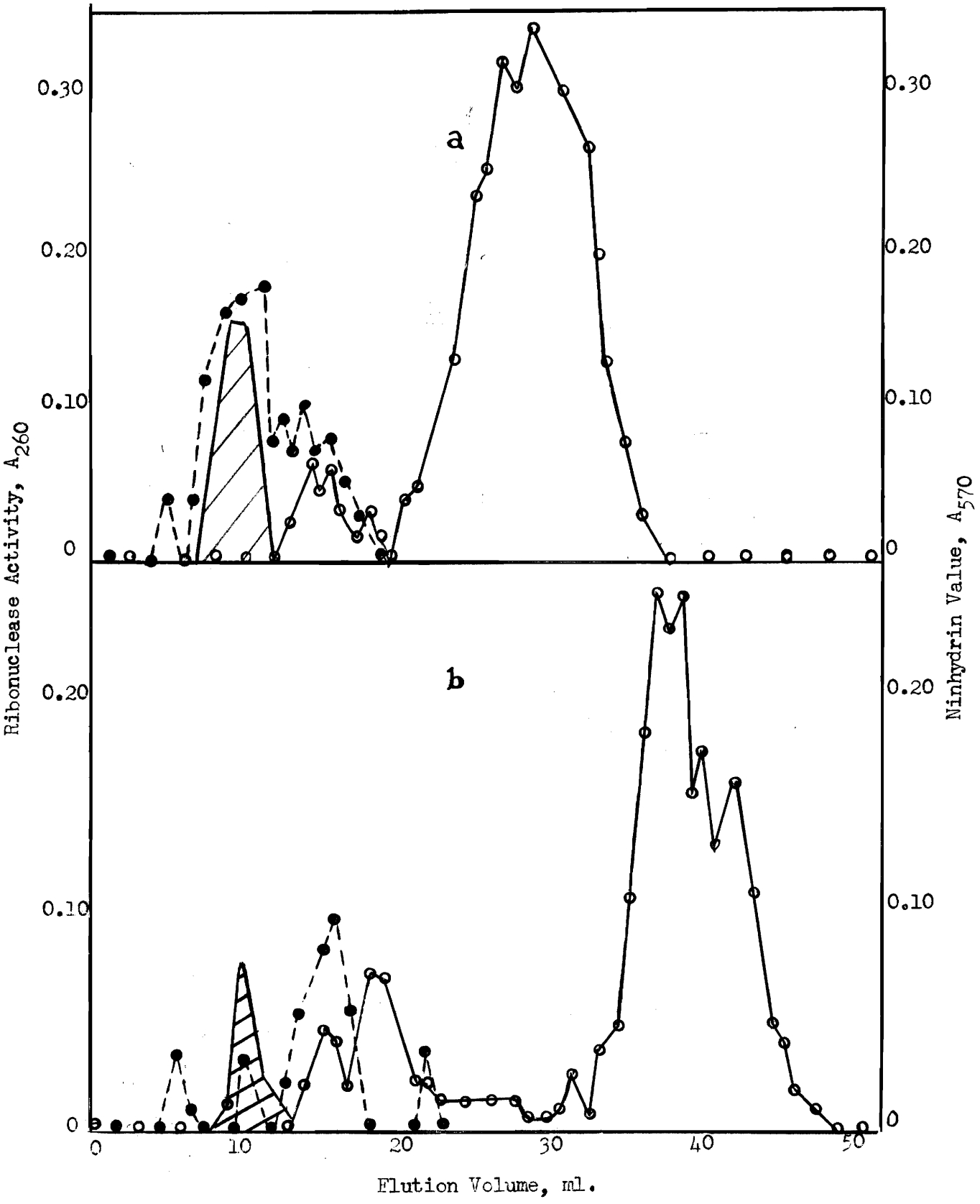
Figure 8. Chromatography of extracts of the principle zymogen granule fraction on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a sodium phosphate buffer extract of the zymogen fraction; b, a curve obtained from a Hirs et al. extract of the same cell fraction. ●, ninhydrin value; O, ribonuclease activity assayed at pH 5.0.


0.261
a
b
0.20
0.15
0.10
0.05
0
0.20
0.10
0
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Values, A_{570}
Elution Volume, ml.

microsomal fractions. For this reason, the ribonuclease rich (text - Fig. 6) microsomal fraction (cytoplasmic f) was chromatographed independently and parallel studies were carried out on combined aliquots of the cytoplasmic fractions g and h, the high speed microsomal fractions.

a. "Cytoplasmic f" microsomal fraction - A comparison of the chromatographic elution curves obtained from the whole-tissue extracts and those obtained from extracts of the "cytoplasmic f" microsomal subfraction (Fig. 9) indicates several striking similarities: namely, (a) the material of the main peak of the non-treated microsomal extract (i.e., the hump at elution volumes 25 to 45) appears to be chromatographically indistinguishable from the material designated as the "III" component from the whole-tissue extracts. This finding, when compared to the sharp, symmetrical "III" component present in the nuclear and zymogen fractions, suggests that two or more closely related ribonucleases may be involved in this broad zone of enzymatic activity from both the total tissue and microsomal fraction studies. In addition, the shape of the curves of duplicate studies of the non-treated extract of this fraction (see Appendix) reveals the possible presence of variable amounts of similar enzymatically active ribonucleases which contribute to the breadth of the effluent curves between 25 and 50 ml. In addition, (b) the data indicate that both the Hirs et al. and the phosphate buffer preparations of the "cytoplasmic f" microsomes contain amounts of peak III similar to that present in the whole-tissue extracts. In the sulfuric acid treated microsomal fraction, however, the peak III appears to be more than doubled either through a direct activation of a "latent" ribonuclease, or a conversion of the front-

Figure 9. Chromatography of extracts of the cytoplasmic f microsomal subfraction on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a sodium phosphate buffer extract of the cytoplasmic f microsomal subfraction; b, a curve obtained from a Hirs et al. extract of the same cell fraction. ●, ninhydrin value; 0, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.


a
b
0.30
0.20
0.10
0
0.20
0.10
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Flution Volume, ml.

running portion of peak III during the sulfuric acid treatment of the sample. It follows from these data therefore, that a unique acid labile ribonuclease component, to be designated IIIa is contained solely in the particulate structure of the microsomes while a second acid-stable component IIIb is localized both in the zymogen granules and the extra-granular microsomes. The terminology "acid-stability" or "acid-lability" refers (in these instances) to the chromatographic reappearance of the non-acid treated eluted RNase components on treatment by the Hirs et al. procedure, and does not necessarily indicate an actual loss of enzymatic activity.

It is evident, however, from a comparison of the whole-tissue phosphate buffer extract (Fig. 3) and the corresponding extract of the microsomal fraction (Fig. 9), that peak I is not present in the "cytoplasmic f" microsomes although a very prominent component of 260 mμ absorbing material was eluted in the position of peak I, simulating the elution pattern of the whole-tissue phosphate extract. Most of this 260 mμ absorbing component of the homogenate was in fact, recovered in the cytoplasmic f microsomal fraction (~85 per cent) and in the final supernatant (~13 per cent). This 260 mμ component was present in very small amounts in the nuclear and dense granule fraction and was apparently absent in the zymogen fraction.

b. The "cytoplasmic g" and "h" microsomal sub-fractions - In the high speed microsomal sub-fractions, the concentration of ribonuclease activity was lower than, or equal to, that found in the mitochondrial fractions (Table VII). As already mentioned, it was anticipated that the level of activity in these fractions would be due to contamination

from the cytoplasmic f microsomes. In this respect, Siekevitz and Palade (47) have in fact, shown that the corresponding sub-fractions from guinea pig pancreas consist almost entirely of detached RNP particles and are morphologically distinct from the initial microsomal fraction. To test this hypothesis, chromatographic studies were carried out on phosphate buffer extracts of combined aliquots of fractions g and h (see text - "Methods"). The analytical data is shown in Figure 10. It is evident from the data in Table IX and the chromatographic evidence in Figure 9 that the enzymatic homogeneity of the non-acid treated microsomes can be questioned on three main grounds: namely, 1) the presence of peak I in the cytoplasmic g and h fractions, and not in cytoplasmic fraction f; 2) the absence of peak II in the high speed g and h fractions, compared to cytoplasmic fraction f, and finally, 3) the apparent heterogeneity and rapid elution of a broad peak appearing at elution volumes of 20 to 30 ml. An interesting difference in the structural relationships of these particles is indicated by the total lack of a 260 mμ absorbing component eluting at 10 ml. in these lighter cellular fragments.

The cell "soluble" or supernatant fraction - Of the total activity of the homogenate, 15 to 20 per cent (a figure comparable to that found for other soluble enzymes) was recovered in the final supernatant. Part of these activities undoubtedly originate from damaged granules or microsomes, but it is also possible that small amounts of the ribonucleases pre-existed in soluble form in the intact cell. To test this latter possibility the final supernatant from three comparable cell fractionation studies were chromatographed under the conditions given in the experi-

Figure 11. Chromatography of extracts of the final supernatant (S-1) from the cell fractionation studies on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a non-acid treated supernatant sample; b, a curve obtained from a Hirs et al. extract of the same final supernatant. ●, ninhydrin color value; 0, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.

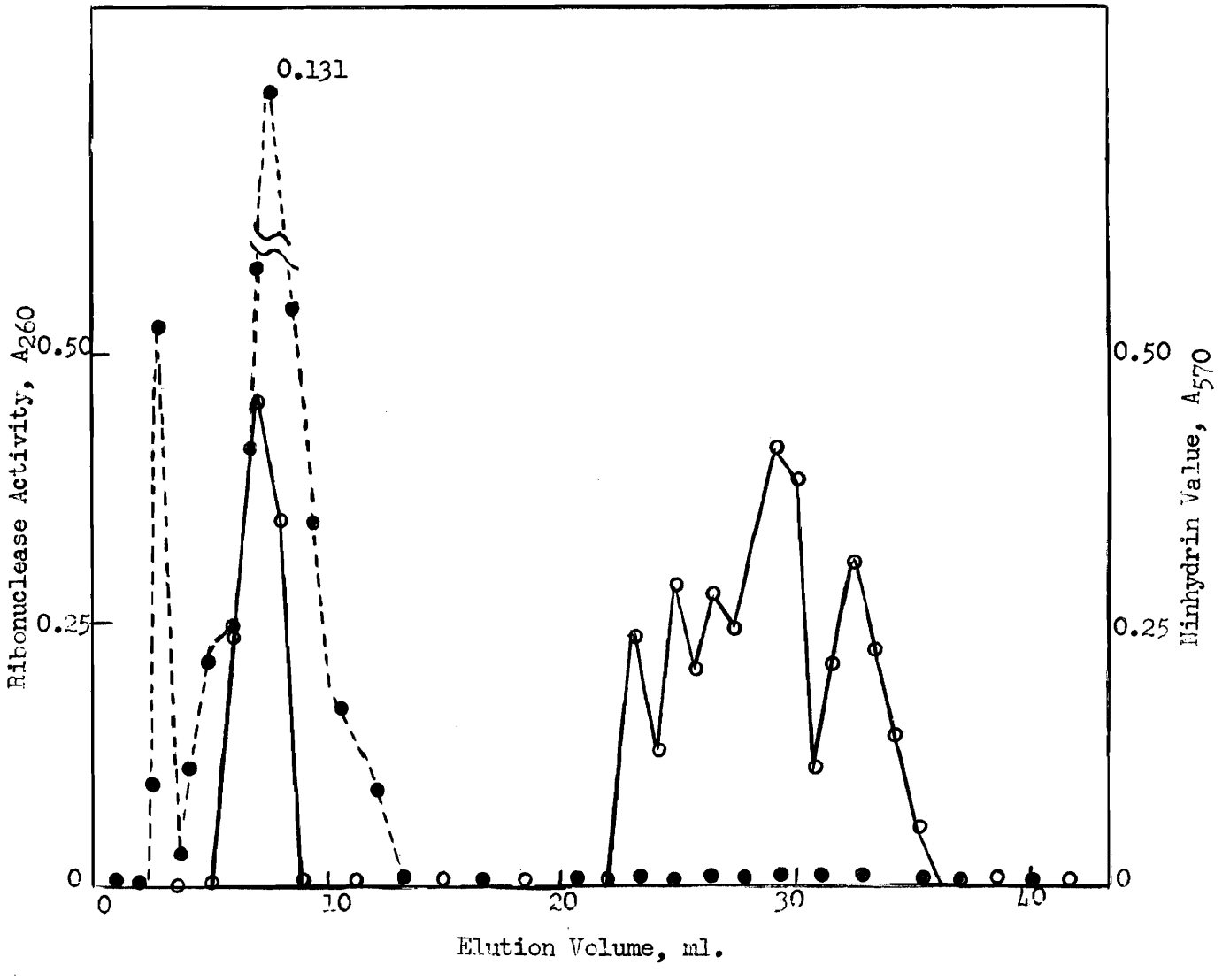
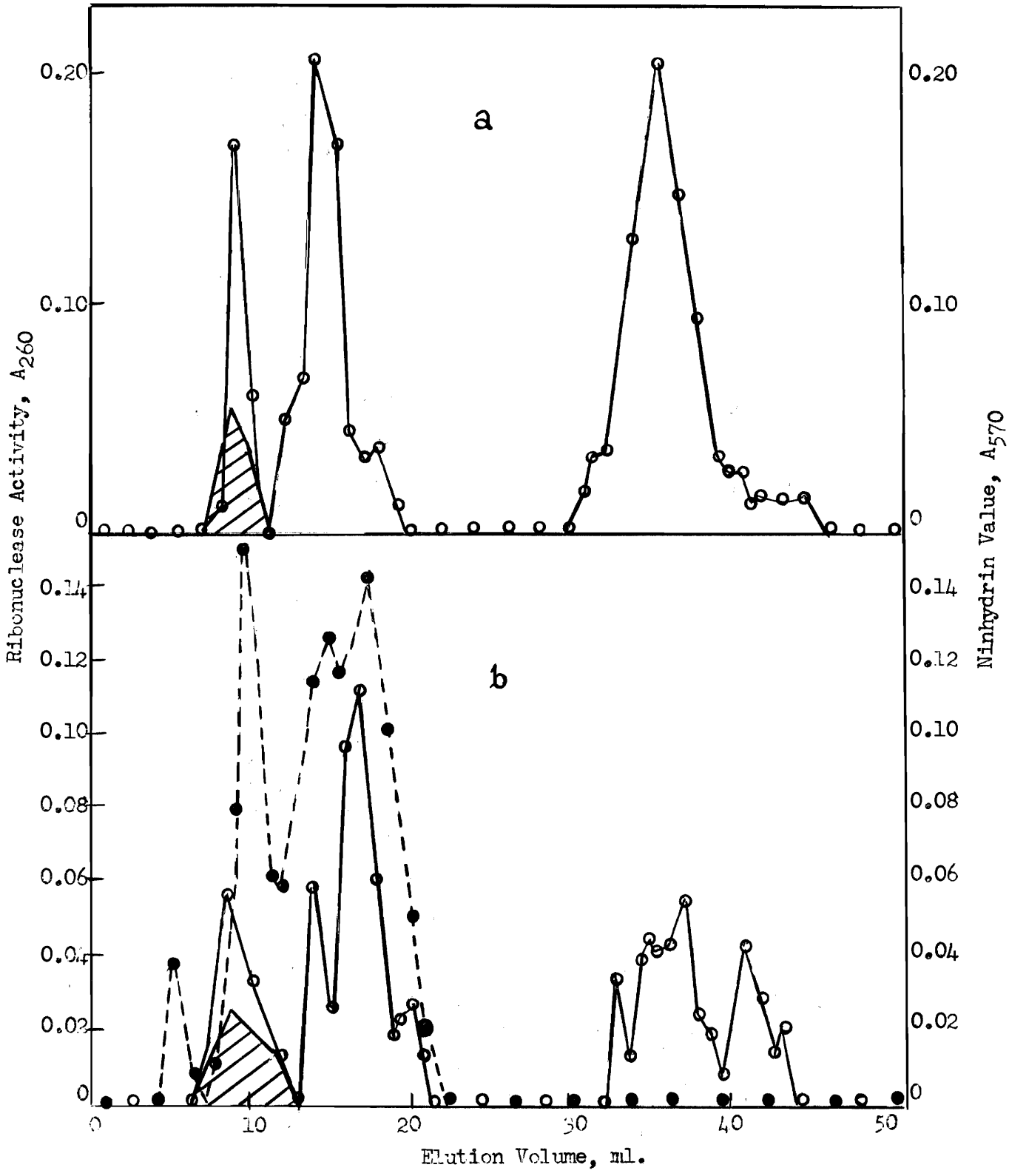


Figure 10. Chromatography of a sodium phosphate buffer (0.2 M) extract of combined microsomal subfractions g and h on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer as eluent. For details of the procedures see text under "Methods". ●, ninhydrin color value; O, ribonuclease activity assayed at pH 5.0.

mental section. The data in Table IX and Figures 11, 12, and 13 indicate clearly that the final supernatant is markedly heterogeneous and enzymatically variable at the resolution level of the chromatographic methods used. These chromatographic patterns exhibit several noteworthy features; (a) as mentioned earlier in this section, the "soluble" portion of the cell is the only fraction containing component I of the non-treated tissue extracts. This finding in fact, substantiates the afore mentioned proposition that at least small amounts of the ribonucleases pre-existed in soluble form in the intact cell, at least as far as this one component is concerned. (b) The remaining enzymatically active ribonuclease components from non acid-treated mouse pancreas demonstrated no consistent pattern in the four supernatants studied. Component IIIb for example, was found to be almost entirely absent from two of the four experiments, while the IIIa component could not be detected in any of the supernatant samples examined. (c) As had been suggested by earlier results, peak II from the non-treated extracts now appears to involve two and possibly three closely related and enzymatically active ribonuclease components. Extracts of supernatant 1, for example, gave an elution curve for peak II similar to that of the whole-tissue extracts of mouse pancreas. However, in one experiment (Fig. 13a), there was evidence of a second component immediately following the usual placement of peak II and representing, in the whole-tissue extracts, only a small trailing hump producing a general asymmetry of this peak. In contrast to these results, in a third study (Fig. 13b) this slow moving portion of peak II became the major component comprising more than 70 per cent of the activity eluted from the supernatant, and

Figure 12. Chromatography of extracts of the final supernatant (S-2) from the cell fractionation studies on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a non-acid treated supernatant sample; b, a curve obtained from a Hirs et al. extract of the same final supernatant. ●, ninhydrin color value; 0, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ, absorbing component.


a
b
0.20
0.10
0
0.14
0.12
0.08
0.06
0.04
0.02
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Elution Volume, ml.

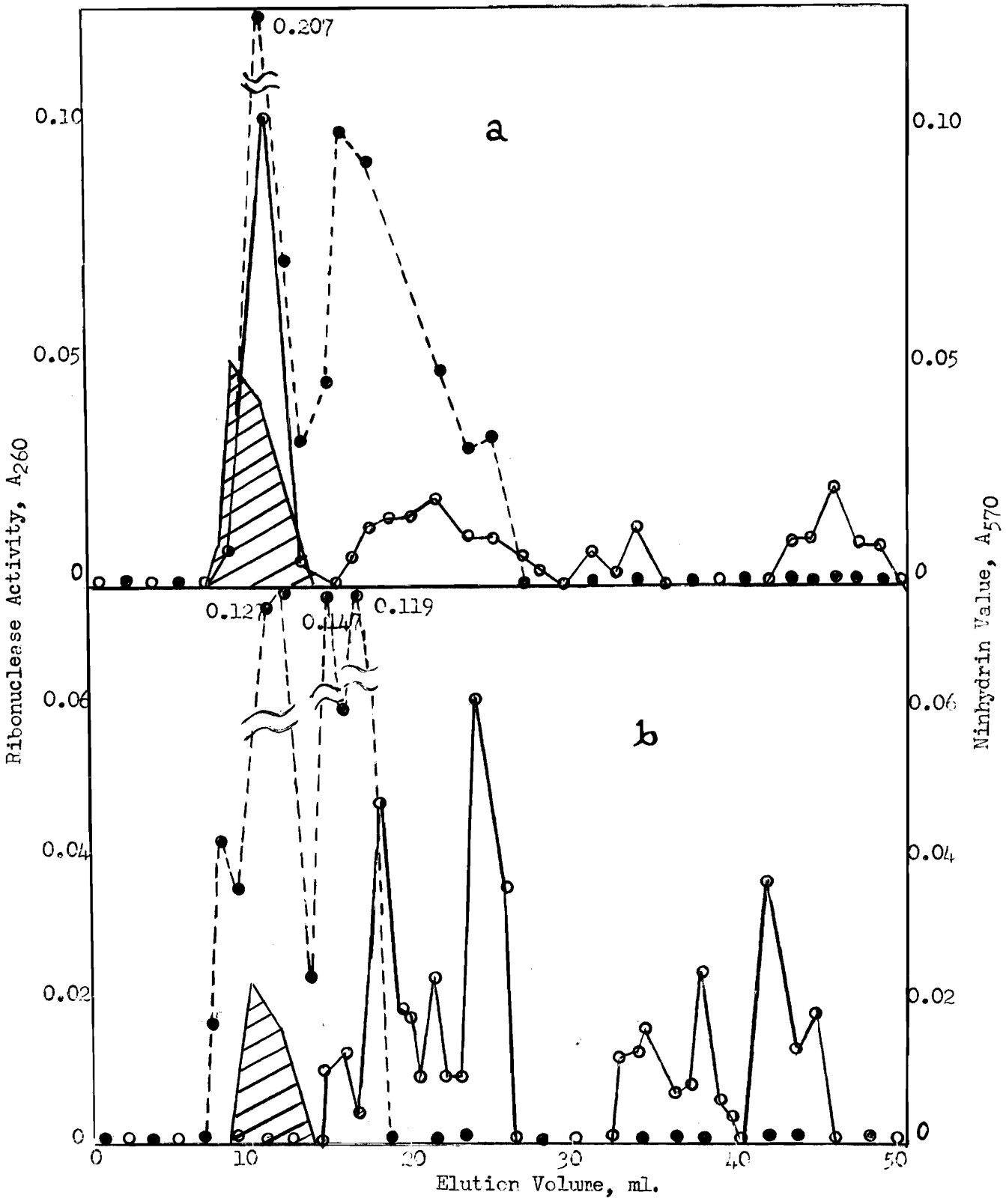

0.207
a
0.10
0.05
0
0.10
0.05
0
0.127
0.144
0.119
b
0.06
0.04
0.02
0
0.06
0.04
0.02
0
0
10
20
30
40
50
Elution Volume, ml.
Ribonuclease Activity, A260
Ninhydrin Value, A570

Figure 13. Chromatography of non-acid treated extracts of final supernatants S-3 and S-4 from the cell fractionation studies on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. ●, ninhydrin color value; O, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.

thus localized 90 per cent of this portion of peak II in the untreated pancreas. A fourth interesting difference was found in the final supernatant study (Fig. 12), in which peaks II and III were almost totally absent, the single enzyme present being the now universal (in the supernatant) component I. And finally, (d) treatment of these samples by the method of Hirs et al. (Figs. 11a and 12a) produced such a heterogeneous elution pattern as to be completely uncorrelatable to other fractions receiving corresponding treatment.

The enzymological variations thus described cannot be correlated with any obvious difference in the extraction or the preparation of the samples, but must be explained in respect to a delicate physiological balance within the secretory cell. This situation will be discussed later in greater detail.

Recovery of ribonuclease activity from the chromatographed fraction - An interesting difference, which concerns the total enzymatic activity eluted from the various cell fractions, emerges when the experiments on the initial nuclear and zymogen fractions are compared with those carried out on the microsomal and soluble fractions. As shown in Table X, in the case of these latter fractions, a two to five fold increase in ribonuclease activity was recovered in the column eluates compared to a nearly quantitative recovery from the nuclear and zymogen fractions. The reason for the greater than 100 per cent recovery is not known. It might be explained by assuming that the extraction was adequate for the separated fractions through column elution, but not entirely satisfactory when the intact fractions were assayed. However, the reliability of the assay

system can be further questioned because the figure obtained by summing the effluent activities of each and every fraction is considerably greater than the figure found for the effluent activity of the whole homogenate. Hokin has made a similar observation for tryptic activity in the pancreas and explained it by assuming the presence of a trypsin inhibitor in the homogenate (41). A similar explanation may be advanced for the over-recovery obtained in these experiments. If it is assumed that an inhibitor is present in the original homogenate and that its distribution pattern among cell fractions is different from that of the enzymes, then over-recovery is to be expected, especially if most of the inhibitor separates in one fraction and the enzyme in another. However, whether these differences are due to some subtle interactions of the enzymes themselves, structural anomalies in the particulates involved, inhibitors, or combinations of two or more of these possibilities, no adequate explanation can be offered at this time but must await more definitive experiments.

Intra-cellular localization of ninhydrin positive components - In addition to the main findings, which concern the intra-cellular distribution of ribonucleases in the secretory cell of mouse pancreas, some other points in these experiments deserve to be noticed. For reasons mentioned in a preceding section, certain effluent components (i.e., the ninhydrin positive material eluted with the I and II peaks of both extracts, and the 260 mμ absorbing material eluted exclusively with peak I) were assumed to be contaminants from the tissue extracts and not directly related to the enzymes in question. In this respect, the 260 mμ absorbing material has already been shown to be localized almost exclusively in the cytoplasmic f

microsomal sub-fraction, with very small amounts appearing in the nuclear and soluble fractions. In the case of the ninhydrin positive material, however, it was found to be largely localized (~80 per cent) in the soluble portion of the cell, with the remainder being about equally distributed throughout the remaining fractions. One may wonder, however, whether with the unique localization mentioned, these components actually represent specific cellular entities, or are merely a heterogeneous residue from disrupted cells.

III. Response of Cellular Amylase and Ribonuclease Activity to Physiological Changes in the Pancreas.

In order to resolve some of the complicated questions on pancreas metabolism, particularly concerning the function and interactions of the ribonuclease system, additional investigations on the enzymes were carried out during various phases of the secretory cycle. Further studies on the chromatographic behavior of the mouse pancreas "ribonucleases" as well as additional properties of the enzyme amylase, will be reported in this section.

A. Effect of Fasting on Amylase and Ribonuclease Activity in Mouse Pancreas -

1. Enzyme levels in the pancreas during fasting - In connection with the large changes in enzyme activity occurring during the cycle of synthesis and secretion, it was of interest to know whether changes in two enzymes were concurrent and what intracellular enzymatic fluctuations were responsible for these changes. For this purpose, two separate experiments were carried out. Each experiment involved 18 male C57 black male mice, four months of

age, consisting of two sets of litter mates born within the same two week period. In these studies, each group of mice was fasted in starvation cages for the periods of time indicated, but were allowed to have water. Those killed immediately were designated as "zero hour" controls; the rest were killed in groups of three at the indicated intervals after the beginning of the starvation period.

A typical experiment in which the changes in amylase and ribonuclease activity were measured during a period of fasting is depicted in Figure 14.

1. In all experiments, the variable nature of the fasting levels of these hydrolytic enzymes are apparent. In the case of both ribonuclease and amylase activity, a maximal level was reached within 12 hours after initiating fasting and was followed by a decline in activity until a minimum was reached at about 24 hours.

2. Although variations were observed in the relative activities of these enzymes from one experiment to the next (see Appendix for data), on the whole, the large variations in activity during fasting came at about the same time for both amylase and ribonuclease and were consistent within a given experiment. Variability in pancreatic enzyme concentration among control and fasted groups from different experiments must, at least for the present, be attributed to inadequate experimental control of factors pertinent to the process.

3. Finally, a comparison of the curves shows that when mice are fasted for 24 to 30 hours (a prolonged fast for a small animal having a high rate of metabolism), the activities of both enzymes are reduced to a low level. Under optimum conditions the extent of this decrease in

Figure 13. Chromatography of non-acid treated extracts of final supernatants S-3 and S-4 from the cell fractionation studies on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. ●, ninhydrin color value; O, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.

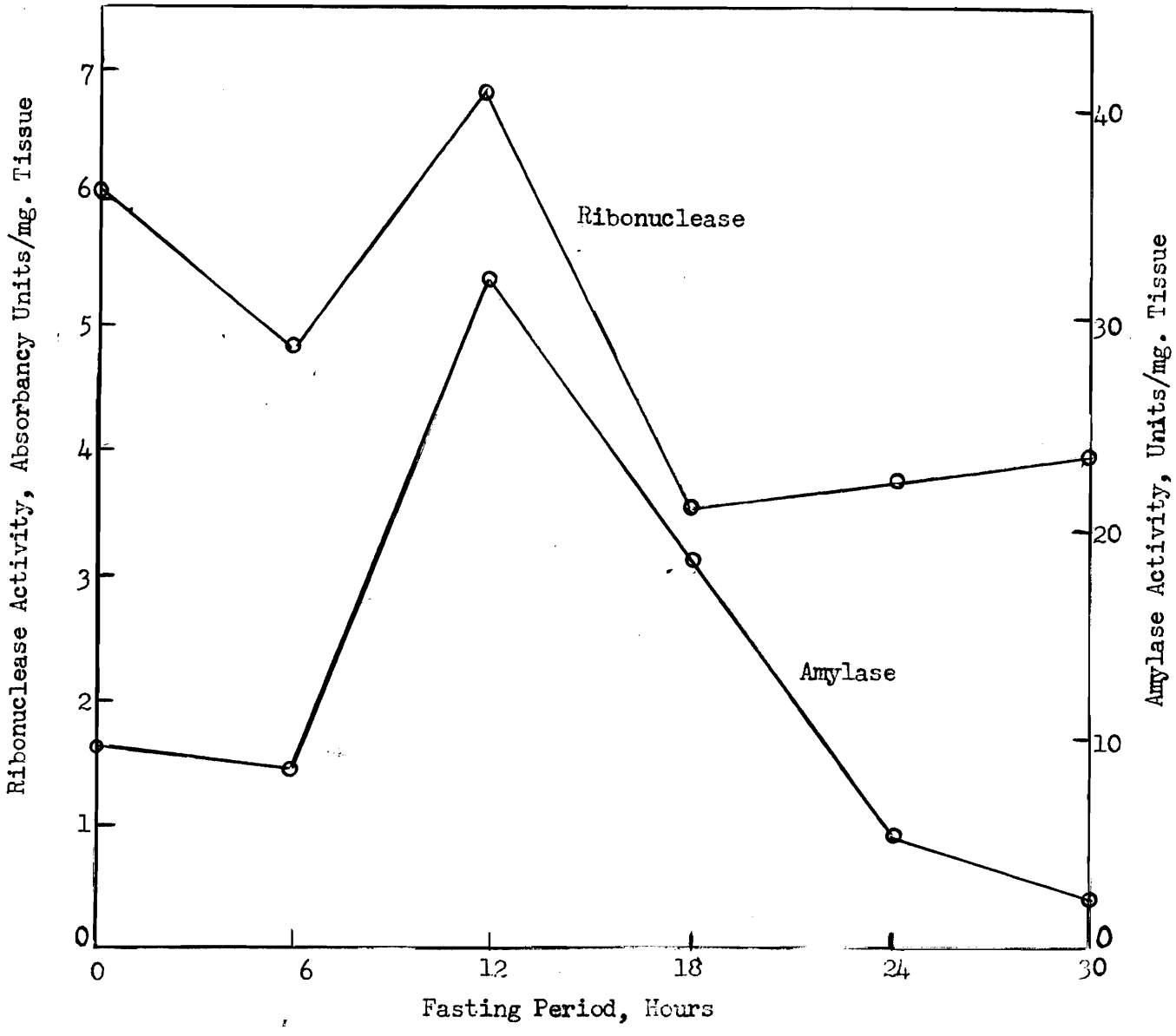


Figure 14. Ribonuclease and Amylase Activity in Mouse Pancreas as a Function of Fasting Period. Mice fasted for the period indicated, water allowed. Ribonuclease activity assayed at pH 5.0; amylase activity assayed as described under "Methods".

activity can be very large in the case of amylase (~70 per cent) and relatively less pronounced for ribonuclease (~30 per cent). Full control is difficult to attain in animals in which stimulation to secretion and therefore synthesis is governed by a complexity of internal conditions. Age and strain of mouse must be very carefully controlled in a given experiment or a series of experiments. Best results in fact, are obtained from litter mates. It is also to the point that it is difficult to syncronize the animals to a point for initiating fasting, for it is difficult to standardize the time of ingestion or the amount of food taken by the mice even when they have been previously fasted.

2. Chromatography of extracts of fasted tissue - From the standpoint of the present objectives, the main interest lies not in the magnitude of the fasting enzyme levels per se, but in the behavior of certain intracellular components (and their associated "nucleases") which must somehow be associated with the changes described above.

The previous studies with ribonuclease (Fig. 2) demonstrated that the IRC - 50 columns are particularly well suited for chromatographic analyses of ribonuclease activity in crude mouse pancreas extracts prepared by the method of Hirs et al. For this reason, it was anticipated that a study of the chromatographic behavior of ribonuclease from fasting pancreas would give an indication of the fractions involved in the increase in ribonuclease activity at 12 hours, and the subsequent minimal activity on prolonged fasting. In these experiments, Hirs et al. extracts were prepared from the "0", "12", and "24" hour fasted tissue homogenates and chromatographed as outlined under "Methods". The columns were run at pH 6.47 and

the effluent fractions were surveyed for ribonuclease activity and for ninhydrin positive material. The results are shown in Table XI and Fig. 15. Extracts of mouse pancreas from animals subjected to either 12 or 24 hour fasting periods gave a picture similar to that for extracts of non-starved mouse pancreas (Fig. 2) with a few differences: namely, (a) From the data presented in Table XI, peak III appears to have lost ribonuclease activity. This loss in turn could account for the overall 30 per cent decrease in ribonuclease activity during prolonged fasting, but would hardly explain the increase in activity at 12 hours. (b) In general, however, the material of the two major peaks from acid extracts of fasted pancreas was chromatographically indistinguishable from the corresponding material designated as the II and III components of Figure 2. And finally, (a) the chromatograms again showed the presence of non-ribonuclease substances eluting in the region of peaks I and II. These ninhydrin positive components have more than tripled in total concentration on fasting and in the case of the 12 hour chromatographic studies began to come off the column as soon as the hold-up volume was reached. These substances (including the 260 mμ absorbing material eluted at 10 to 12 ml.) could be removed by dialysis, however, and the results of a chromatogram performed upon a dialyzed extract are shown in Figure 20.

B. Secretion (Active Extrusion) of Amylase and Ribonuclease in Vitro -

1. Reliability of the in vitro incubation technique - The procedure for releasing stored enzymes and other organic matter from the ducts, acinar lumens, and exocrine cells of the pancreas yielded a basal (control) secretory level from the excised gland which made it possible to obtain

Table XI

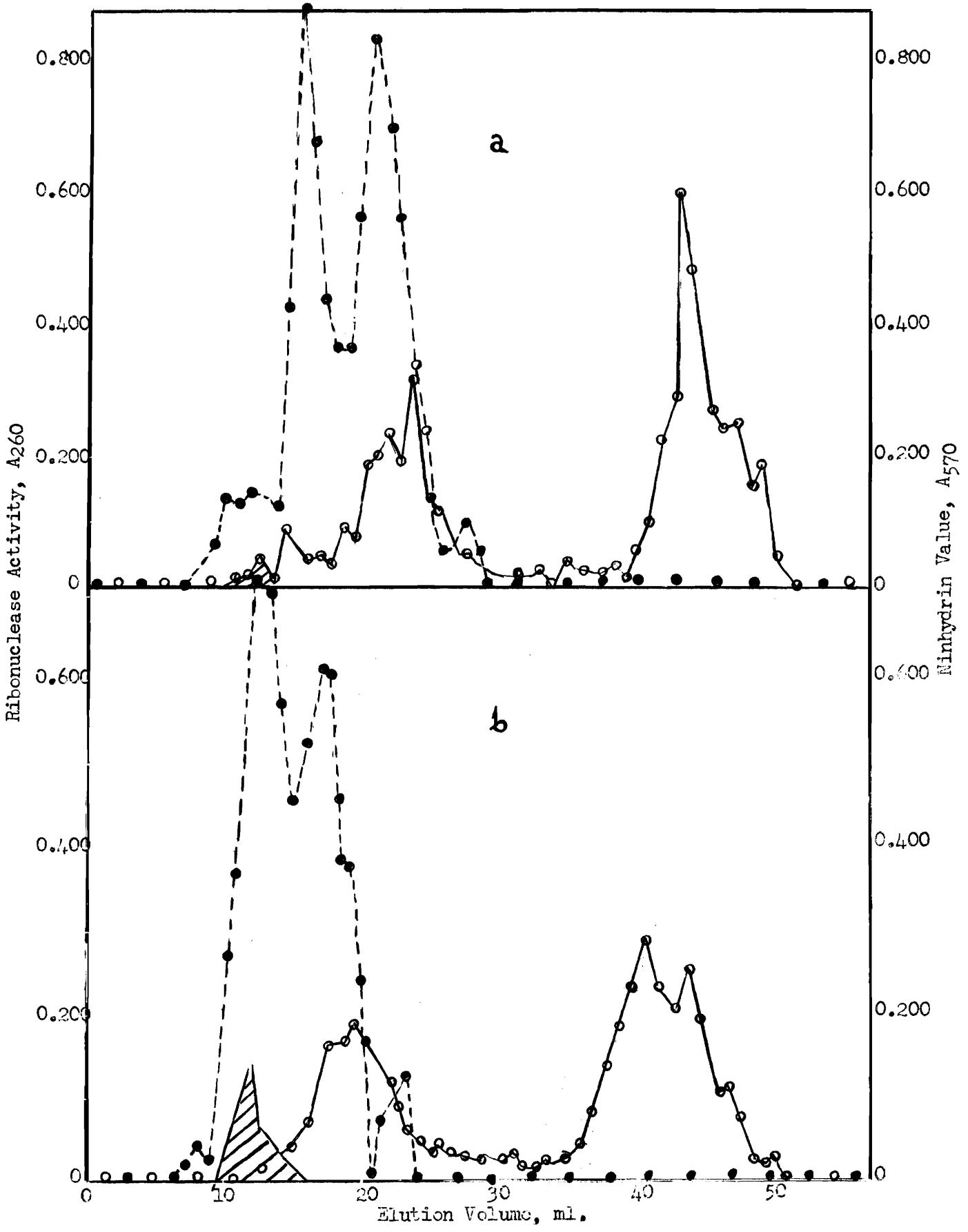
Chromatographic Distribution of Ribonuclease Activity from Fasted and Non-fasted Mouse Pancreas

Pancreas extracts prepared and chromatographed by the method of Hirs et al. Ribonuclease activity assayed at pH 5.0.

Fasting Period	Per Cent of Total Eluted RNase Activity*		
	Peak I	Peak II	Peak III
Control (0 hour)	2	28	70
12 hours	5	37	55
24 hours	8	33	55

* Values averaged from duplicate studies.

Figure 15. Chromatography of 0.25 N sulfuric acid extracts of fasted mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from 12 hour fasted mouse pancreas; b, curve obtained from 24 hour fasted mouse pancreas. ●, ninhydrin value; O, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.


a
b
0.800
0.600
0.400
0.200
0
Ribonuclease Activity, A260
Ninhydrin Value, A570
0
10
20
30
40
50
Elution Volume, ml.

reproducible results of a degree seldom witnessed in an *in vitro* system. In order to obtain such results, however, it is necessary to be very meticulous in regard to the experimental conditions involved.

The experience gained in the present studies suggests that the following points be considered in carrying out *in vitro* studies on the exocrine system of the pancreas:

(a) Strain and age must be carefully controlled. As mentioned under "Methods", the results were all obtained with pancreas from CBA mice. When similar experiments were performed with pancreas from either male C57 black mice, or albinos, quite different results were obtained. The most striking difference was found to be in the amount of enzyme secreted into the control flasks. Instead of an average of about 5 per cent of ribonuclease and amylase secreted into the incubation medium, which was the amount found with CBA pancreas, 10 to 20 per cent was secreted by the C57 or albino pancreas with the actual amount varying widely from one experiment to the next. With respect to age, a range of two to four months is essential, with the age of the mice in any given experiment not varying by more than two weeks. Pancreas from mice less than two months old have high respiration quotients and increased enzyme secretion in the control flasks, while on the other hand, tissues from older animals may not respond optimally to release stored enzymes.

(b) The animals must be killed immediately by cervical dislocation in the manner described under "Methods". Decapitation increases the control enzyme level to 20 per cent into the medium. Anesthesia has an immediate and remarkable effect to depress respiration and makes the

tissue refractory to *in vitro* stimulation (Table XII).

(c) A carefully controlled pretreatment of the excised tissues is a necessary prerequisite for reliable and reproducible results. First the gland must be removed from the animal and handled with a minimum of forcep pressure and manipulation. Secondly the excised tissues are pre-cooled to an optimum temperature of 8° for a 10 to 15 minute period before transferring to the 37° Warburg bath. And finally, the flasks should be gassed with the oxygen:carbon dioxide mixture for a maximum of two minutes at a pressure of 6 pounds per square inch. A volume flow greater than this will result in respiratory stimulation and increased release of enzyme into the medium.

However, despite the necessity for meticulous attention to minute details, the results of the present study indicate that this procedure can give reproducible results not only within a given experiment, but within a series of experiments.

2. *Effect of aerobic and anaerobic incubation on enzyme secretion* - Mouse pancreas released enzyme to the medium during an *in vitro* incubation in the presence of pancreozymin or pilocarpine. As shown in Table XIII, this loss could be reduced to the level of the control flasks under anaerobic conditions. Most of the enzyme found in the medium after aerobic incubation appeared, therefore, to have been extruded by an energy requiring process rather than to have been lost from the tissue by passive diffusion from intact or damaged cells.

Table XII

Effect of the Method of Killing on in Vitro Respiration and "Active Extrusion" of Ribonuclease Activity in Whole Mouse Pancreas

All tissues were incubated for two hours in Kreb's III buffer. For details of the procedure see the experimental section. Ribonuclease activity assayed at pH 7.5 by the standard procedure. Data are average values for duplicate studies.

Method of Killing	Per Cent RNase in Medium		$-Q_{O_2}$	
	Control	Stimulated 0.1 mg. % Pancreozymin	Control	Stimulated 0.1 mg. % Pancreozymin
Cervical dislocation	5.3	23.4	6.3	10.5
Anoxia (nitrogen)	14.3	18.7	10.0	8.3
Anesthesia (ether)	8.3	11.3	5.1	6.3
Sodium Pentothal	16.3	14.1	6.7	8.1
Decapitation	14.6	15.7	6.1	6.5

Table XIII

Effect of Anaerobisis on the "Active Extrusion" of Mouse Pancreas Ribonuclease under Conditions of in Vitro Stimulation

The tissues were incubated for two hours in Kreb's III buffer by the standard incubation procedure (see "Methods"). Anaerobic conditions were attained by flushing a Warburg vessel with nitrogen containing white phosphorus in the center well. Ribonuclease activity determined by the standard pH 7.5 assay. Per cent release based on the activity in the medium compared to the sum of the activity in both the medium and tissue.

Treatment	Per Cent RNase Released into Incubation Medium	
	Aerobic Incubation	Anaerobic Incubation
Non-stimulated control	2.4	0
Pilocarpine stimulation (5×10^{-7} M)	32.	<1
Pancreozymin Stimulation (1.0 mg. %)	24.	<1

3. Secretion of ribonuclease and amylase in vitro - Under optimum non-stimulated control conditions, mouse pancreas released enzyme into the medium during a two hour incubation. However, it was found that under all the conditions used (i.e., fed or fasted mice), this passive loss was proportional to the initial level of enzyme in the tissue, the loss accounting for only 3 to 5 per cent of the amount of amylase or ribonuclease present initially.

Accordingly, experiments were carried out to test whether the amylase and ribonuclease activity of the medium could be increased by the presence of a secretory stimulant, such as pancreozymin or pilocarpine. These results are presented in Figures 16 and 17. Secretion (active extrusion) occurred from the pancreas of both fed and fasted mice, and the secretion of ribonuclease occurred to approximately the same extent from fasted animals as it did from pancreas of fed animals. As shown in Figure 16, pilocarpine maximally stimulated the respiratory rate at a concentration of 5×10^{-6} M, the highest concentration tested. On the other hand, ribonuclease secretion was stimulated only slightly at 5×10^{-8} M and was maximally affected at a concentration of 5×10^{-7} M. Higher concentrations of pilocarpine resulted in a decreased secretion of the enzyme. By comparison, however, for pancreozymin, maximal stimulation of respiration was observed at 10^{-2} mg. per cent* (Fig. 17), and contrary to the results obtained with pilocarpine, a decrease in respiration was found at higher hormone concentrations. Furthermore, maximal stimulation of both ribonuclease and amylase secretion was found at 10^{-1} mg. per cent, with no decrease at concentrations up to

* Since the molecular weight of pancreozymin is unknown, its concentration is expressed as milligram percentage and refers only to the preparation used in this study.

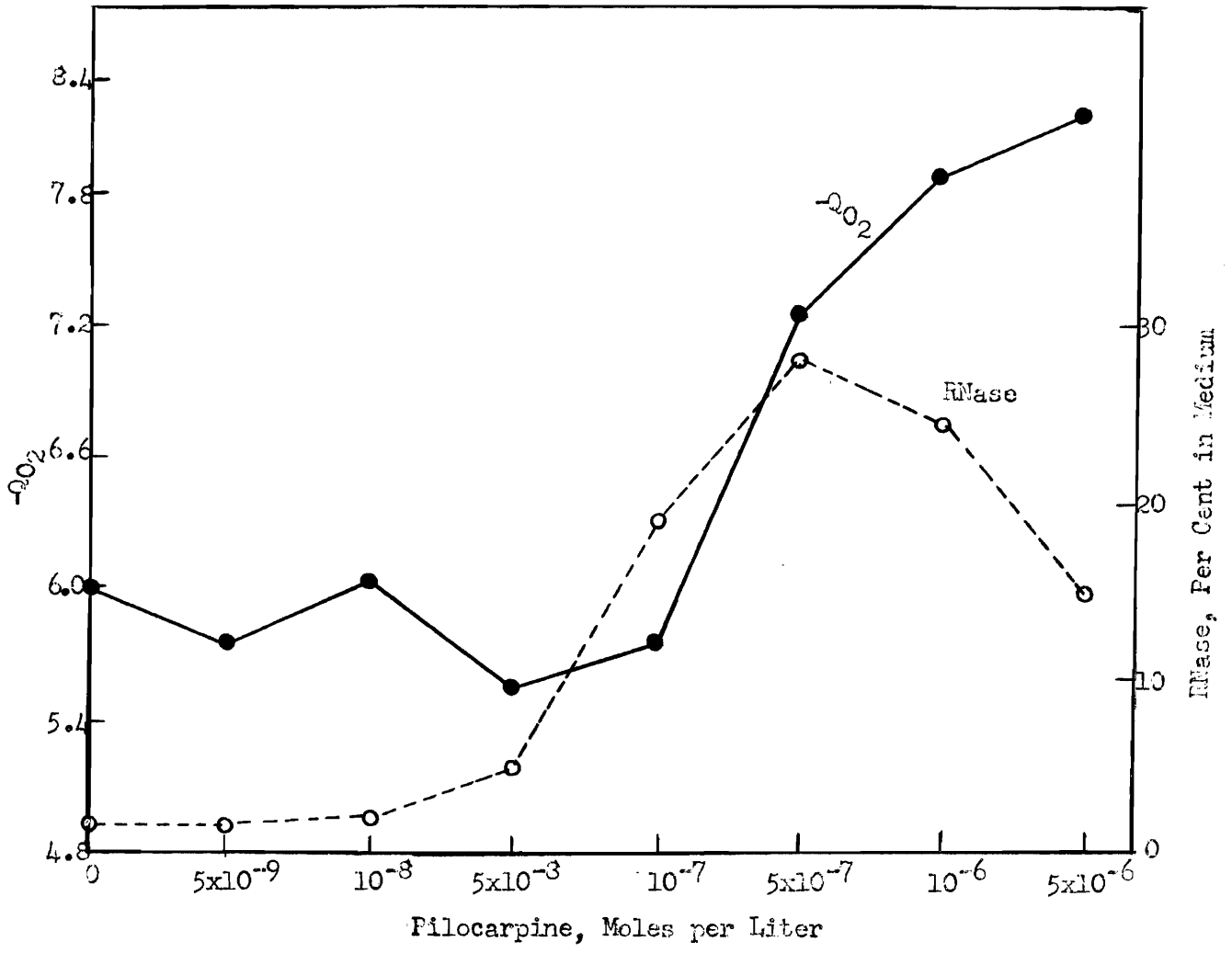


Figure 16. Effect of Pilocarpine on Respiratory Rate and Secretion of Ribonuclease by CBA Mouse Pancreas *in vitro*. Ribonuclease activity determined by the standard pH 7.5 method.

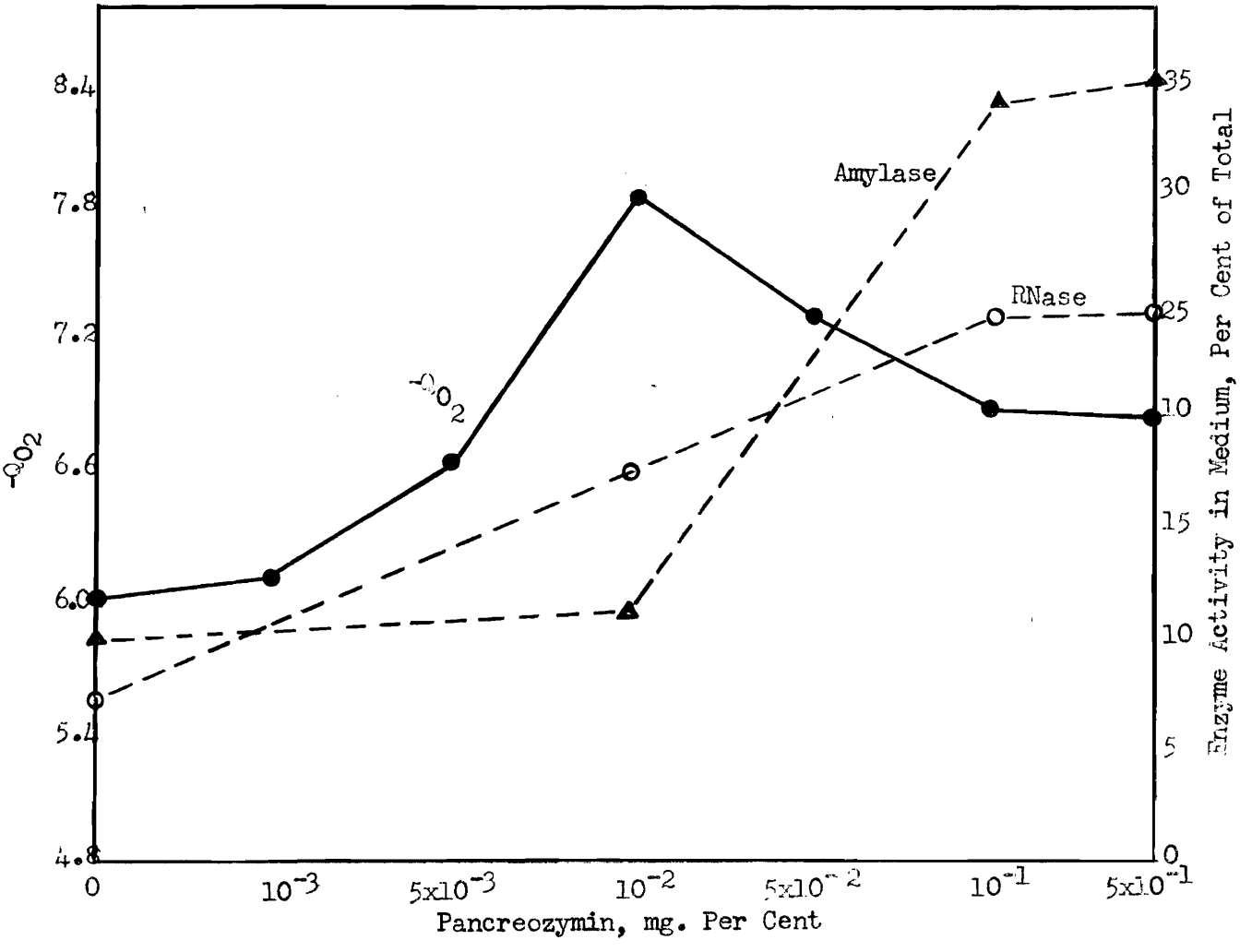


Figure 17. Effect of Pancreozymin on Respiratory Rate and Secretion of Ribonuclease and Amylase by CBA Mouse Pancreas *in vitro*. Ribonuclease activity assayed at pH 7.5. Amylase activity measured as described under "Methods".

1.0 mg. per cent.

C. Chromatography of Ribonuclease Activity Released into the Incubation Medium -

Like pilocarpine, pancreozymin stimulated the release of stored enzymes from the acinar pancreas. From histological evidence, it seems reasonably certain that these stored enzymes are contained in the zymogen granules of the secretory cell. Corell (74) observed, for example, that there was a greater secretion of granules from the pancreatic cells after stimulation by secretin (probably contaminated with pancreozymin) or pilocarpine. More recently the same conclusion was reached in the work of Von Weel and Engel (75) who have likewise demonstrated that stimulation caused a depletion of the granules of the pancreatic cells. Finally, histological evidence (unpublished) from our own in vitro studies indicates that few granules remain in the maximally stimulated tissue. Thus, it seems reasonable to predict, if not actually assume, that there should be a similarity between the chromatographic elution patterns of the "actively extruded" ribonuclease and the corresponding patterns from the zymogen granules of the secretory cells.

Proof for this assumption was provided by the findings presented in Figures 18 and 19. The samples were prepared and chromatographed as outlined under "Methods". The columns were run at pH 6.47 and the effluent volumes were surveyed for ribonuclease activity at pH 5.0 and ninhydrin positive material. On the basis of this evidence it is possible to identify two major enzymatically active ribonucleases in the non-treated incubation medium from both the pilocarpine and pancreozymin studies. These results are similar to those reported for the non-acid treated zymogen and

Figure 18. Chromatography of ribonuclease activity "actively extruded" into incubation medium after stimulation with 5×10^{-7} M pilocarpine on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a sodium phosphate buffer extract of the "pilocarpine incubation medium"; b, a curve obtained from a Hirs et al. extract of the same incubation medium. ●, ninhydrin color; 0, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.

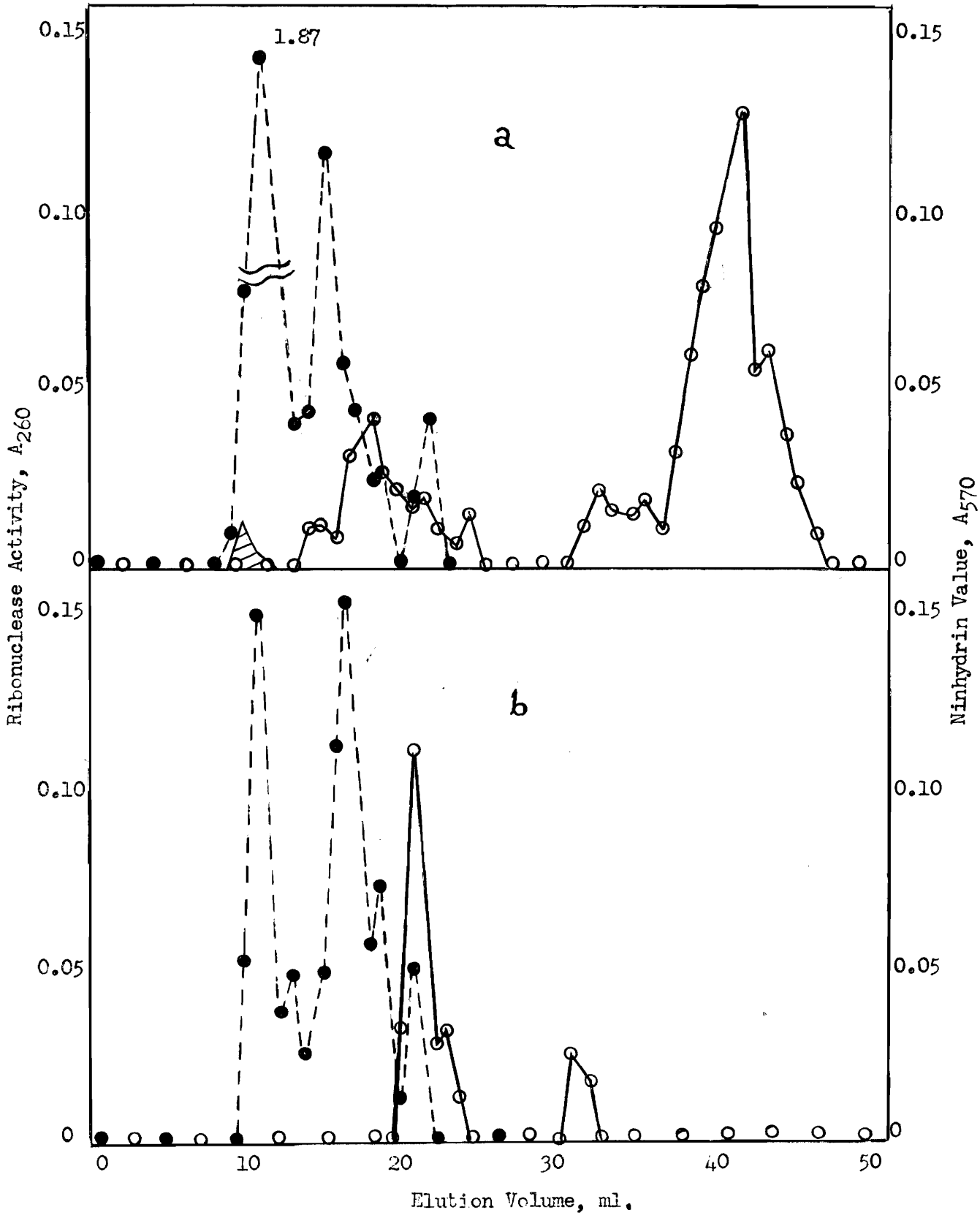
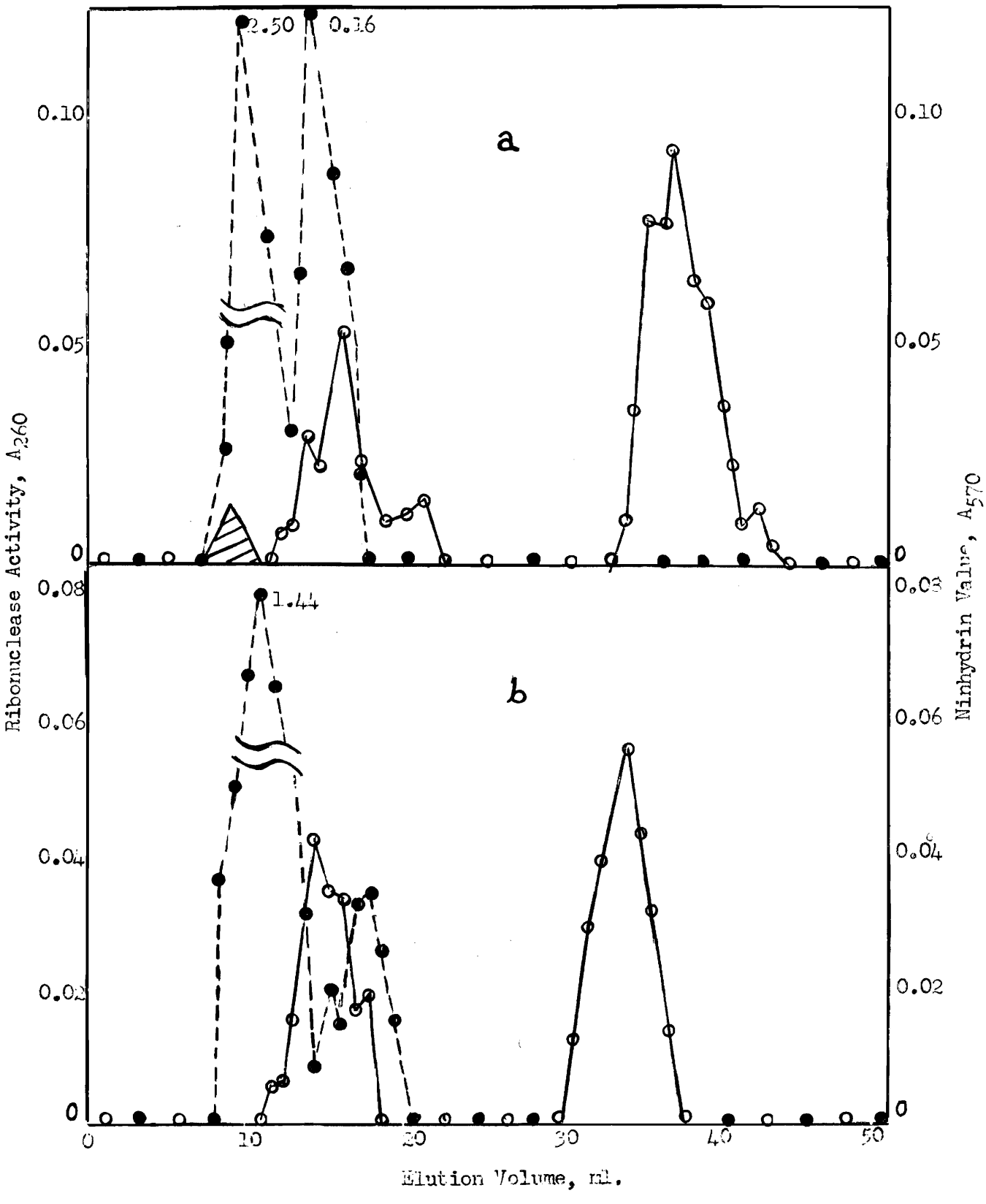

1.87
a
b
0.15
0.10
0.05
0
0.15
0.10
0.05
0
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
0
10
20
30
40
50
Elution Volume, ml.

Figure 19. Chromatography of ribonuclease activity "actively extruded" into the incubation medium after stimulation with 0.1 mg. % pancreozymin on a 9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from a sodium phosphate buffer extract of the "pancreozymin incubation medium"; b, a curve obtained from a Hirs et al. extract of the same incugation medium. ●, ninhydrin color; O, ribonuclease activity measured at pH 5.0. Shaded area, 260 mμ absorbing component.


2.50
0.16
a
b
1.44
0.10
0.05
0
0.08
0.06
0.04
0.02
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Elution Volume, ml.

nuclear fraction elution patterns, in that the major ribonuclease component came off the column at approximately 35 to 40 ml., with a much smaller component coming off at about 16 to 20 ml. Sulfuric acid extracts of in vitro stimulated mouse pancreas gave a quite different picture however. In acid-extracts of the pilocarpine stimulated tissue the major component was totally lacking and the faster moving minor component now appeared as a single symmetrical peak at 20 ml. In addition, in some experiments there were varying amounts of other ribonuclease activity at 16 and 30 ml. In contrast however, the two major components were found in both the acid-treated and non-acid treated extracts of the pancreozymin stimulated tissue. Peak II in this instance became more symmetrical by comparison to the non-treated tissue extract and the per cent of the total effluent activity increased with a corresponding loss in slowly eluting peak III. The data on their distribution is given in Table XIV. Measurements for peak I were not considered reliable enough for tabulation, since a significant level of enzyme activity was not obtained in any of the samples studied. The table clearly shows that a major portion of the enzyme released by pilocarpine is inactivated by treatment with sulfuric acid. Conversely, the enzyme released by pancreozymin shows only minimal effects of acid-treatment. It follows from these data that pilocarpine and pancreozymin selectively release different storage forms of ribonuclease. According to this scheme, it would appear that pilocarpine specifically stimulated the secretion of "nucleases" contained in the heavy secretory granules of the nuclear fraction (Fig. 1), whereas pancreozymin stimulation releases the "nucleases" of the so-called principle zymogen fraction (Fig. 8).

Table XIV

Chromatographic Distribution of Mouse Pancreas Ribonucleases in Medium and Tissue Samples from Stimulated and Non-stimulated in Vitro Incubation Studies

Details of the incubation procedures are given in the experimental section. Distribution was calculated from a summation of the individual eluates of a given peak as per cent of the total activity eluted from the column. Ribonuclease was determined by the standard pH 5.0 assay. Data are average values from duplicate incubation studies. For definition of sample treatment see "Results", page 68.

Treatment	Per Cent Total Eluted Ribonuclease	
	Peak II	Peak III
Incubated Control Tissue		
a. Acid-treated extract	30	70
Pancreozymin Stimulation		
a. Incubation Medium		
(1) Acid-treated	30	70
(2) Non-acid treated	32	68
b. Tissue (acid-extract)	27	73
Pilocarpine Stimulation		
a. Incubation medium		
(1) Acid-treated	83	17
(2) Non-acid treated	24	76
b. Tissue (acid-extract)	25	75

However, one characteristic feature common to both the pilocarpine and pancreozymin studies is the 100 fold increase in the major ninhydrin positive eluting at about 10 ml. By comparison, the two smaller peaks, eluting at 16 and 22 ml. respectively, have not changed. From preliminary studies, the amount of ninhydrin positive material released into the incubation medium appears to be proportional to the degree of stimulation, thus indicating that the liberation of enzymes and the appearance of this material are related phenomena.

D. Chromatography of the Residual Ribonuclease Activity in in vitro Stimulated Mouse Pancreas -

In the preceding sections it has been shown that pilocarpine and pancreozymin stimulation of mouse pancreas in vitro cause a rapid depletion of the zymogen granules of the acinar cells and the appearance of the "storage" digestive "nucleases" in the incubation medium. After a two hour control incubation of mouse pancreas, less than 5 per cent of the total ribonuclease activity is released into the incubation medium. In contrast, the presence of pancreozymin (Fig. 17) and pilocarpine (Fig. 16) produced a release of about 25 and 35 per cent respectively, of the total ribonuclease activity during a similar incubation period. Thus 65 to 75 per cent of the total ribonuclease activity remains within the cell, probably in cellular structures other than the secretory granules.

Limited information on the nature of this residual "nuclease" activity in stimulated mouse pancreas has been obtained from chromatographic elution studies on Hirs, Stein and Moore extracts of these treated tissues. As a control, sulfuric acid extracts of incubated but non-stimulated mouse

pancreas were chromatographed under conditions similar to those described by Hirs et al. (31), and described in this thesis under "Methods". A representative elution pattern of incubated, non-stimulated pancreas is shown in Figure 20. It is evident from these data that the enzymatically active material is chromatographically indistinguishable from similar material eluted from non-incubated, non-stimulated mouse pancreas extracts. Furthermore, there is no increase in the enzymatically inactive ninhydrin positive peaks from the incubated, non-stimulated tissue.

Similar studies with acid extracts of incubated, stimulated mouse pancreas gave little definitive information on the possible intra-cellular changes of the acinar ribonucleases during active extrusion of the stored enzymes. Under the conditions of the Hirs et al. procedure, two enzymatically active components are eluted from the column as shown in Figure 21. The first peak, eluted at two to four times the hold-up volume of the column (12 to 24 ml.) contains a heterogeneous effluent pattern of ribonuclease activity and is eluted somewhat more rapidly from extracts of pilocarpine treated tissue than from corresponding extracts of pancreozymin stimulated pancreas; the second, at about 35 to 40 ml., represents the major portion of the effluent activity. The reason for the asymmetry of the trailing edge of the pancreozymin elution pattern is not apparent.

As previously mentioned, the term acid treatment refers to conditions which cause extensive alteration and a partial inactivation of certain intra-cellular ribonucleases. Thus chromatographic studies on acid-extracts alone cannot define the enzyme system involved and must be largely limited to use as a method for determining the nature of a "nuclease"

Figure 20. Chromatography of 0.25 N sulfuric acid extracts of incubated non-stimulated mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64). a, curve obtained at pH 6.47 with an undialyzed extract; b, curve obtained after dialysis against 0.001 M EDTA for 12 hours at 4° and chromatography at pH 6.47. ●, ninhydrin value; 0, ribonuclease activity measured at pH 5.0. Shaded area, 260 mμ absorbing component.

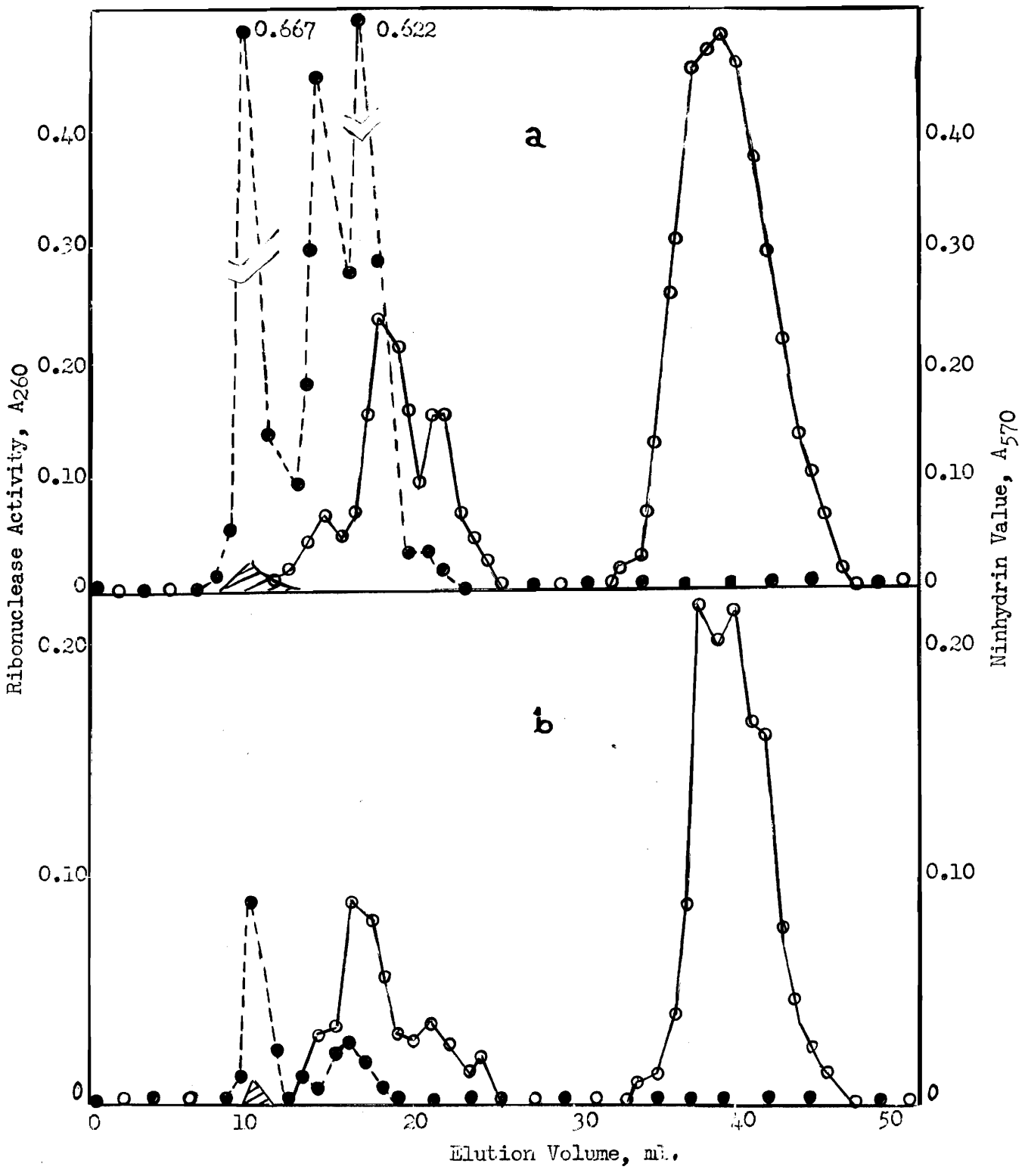
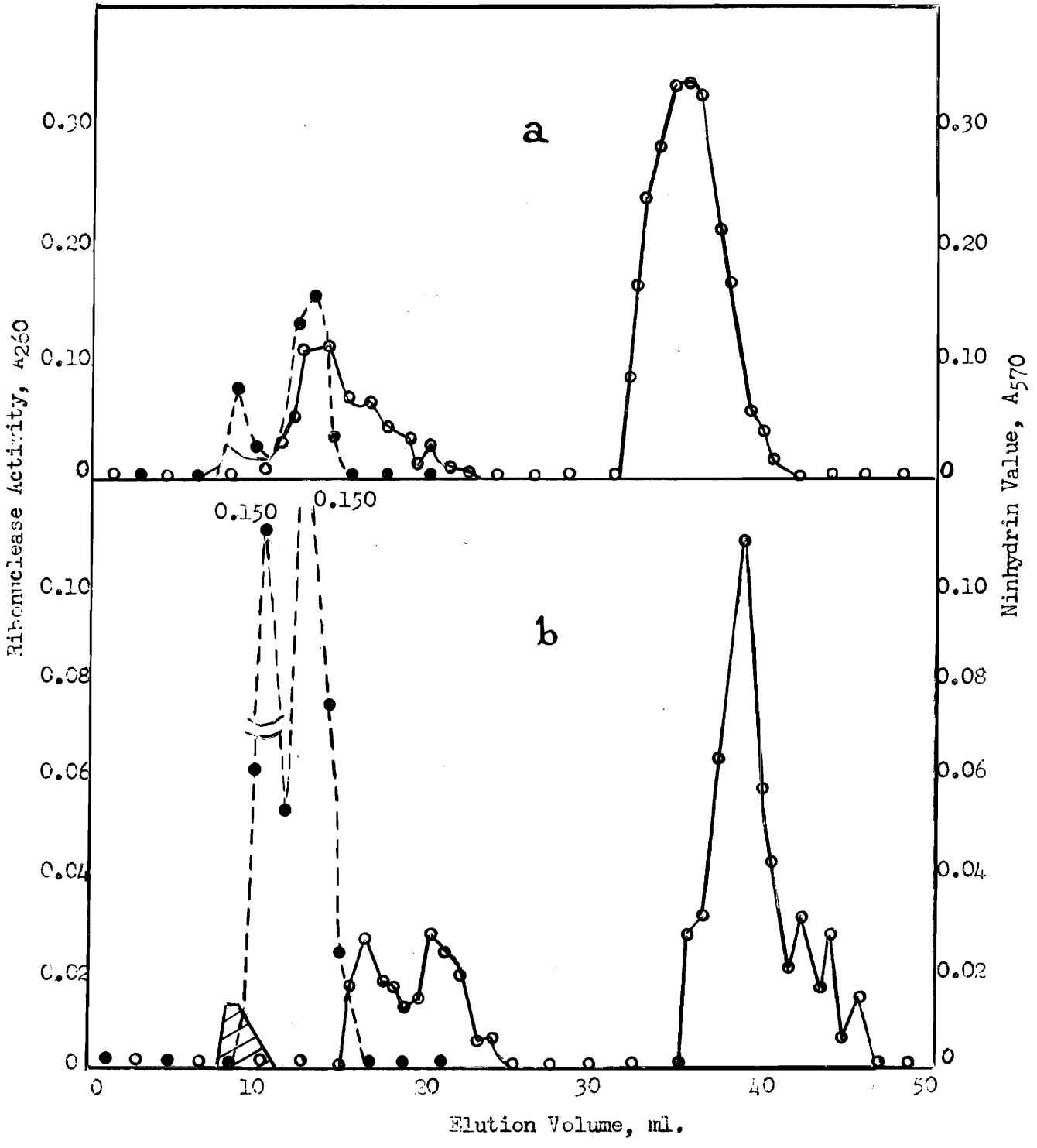

0.667
0.622
a
b
0.40
0.30
0.20
0.10
0
0.20
0.10
0
0
10
20
30
40
50
Ribonuclease Activity, A260
Ninhydrin Value, A570
Elution Volume, ml.

Figure 21. Chromatography of 0.25 N sulfuric acid extracts of *in vitro* stimulated mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer at pH 6.47 as eluent. a, curve obtained from pancreas stimulated with 5×10^{-7} M pilocarpine; b, a curve obtained from pancreas stimulated with 1.0 mg. % pancreozymin. ●, ninhydrin value; O, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing material.


a
b
0.150
0.150
Ribonuclease Activity, A260
Ninhydrin Value, A570
Elution Volume, ml.
0
10
20
30
40
50
0.30
0.20
0.10
0.08
0.06
0.04
0.02

component isolated from non-treated pancreas extracts (i.e., acid-stability or lability). Further information on the intra-cellular variations of the ribonuclease system must await a systematic study of both acid and non-acid treated extracts of isolated cell fractions from stimulated mouse pancreas.

DISCUSSION

The physiological function of ribonuclease in any tissue *in vivo* is not well understood. Thus the significance of two or more ribonucleases in the same tissue or in different tissues is not readily apparent, and there is, therefore, no reason to expect any similarity between the properties of the predominant RNase, or RNases, of various tissues. In respect to the ribonucleases of a specific tissue such as the pancreas, it is somewhat surprising to find wide variations in both the chromatographic heterogeneity and amounts of this enzyme in the same tissue from closely related species. However, through the use of many different assay methods and varying nomenclature, confusion has arisen as to the action of the nucleases and their specificity. In this sense, most of the work published on ribonuclease has dealt with the beef pancreatic enzyme, and in some papers the unqualified word "ribonuclease" is used as if it could only refer to the pancreatic enzyme. This restriction is unreasonable and, because enzymes from different sources have significantly different properties, the enzyme source should always be stated. Thus, for example, an enzyme has been isolated from spleen (17) which shows the same specificity as beef pancreatic ribonuclease and differs only in certain physical properties from the beef enzyme. Conversely, two distinct ribonucleases have been demonstrated in rat liver (20), each quite different in its properties from the other and also differing in varying degrees from the beef

pancreatic enzyme.

Thus in general it can be stated that ribonucleases from various tissues of a single species may have widely different properties, differences in fact more pronounced than those found for the same tissue from several different species. In this respect, it might be expected that the ribonucleases of widely dissimilar organisms, such as animals and plants, would be different in view of differing metabolic requirements, and this has, in fact, been found by Holden and Pirie (76). These workers have shown that ribonucleases from such diverse sources as beef pancreas and pea leaves not only have unique ranges of specificity, but also differ in their rates of hydrolysis of nucleic acid, and in thermostability and pH optimum.

In general however, the similarities that have been found are, in fact, more striking than the differences: in this respect each is more thermostable than most of the other enzymes in the same tissue; the same inhibitors act upon each; and most appear to be relatively small proteins. However, many if not all of these differences are based on the hydrolytic action of these enzymes on nucleic acid substrates. On the basis of data from several workers (77,78), we might question the assumption that free nucleic acid is a normal component of tissues so that these studies may have no immediate relevance. It would be convenient, therefore, if these differences could be correlated with other actions of the enzyme before an attempt is made to assign a function or functions to ribonuclease *in vivo*.

I. The Nature of the "Nucleases" of Mouse Pancreas -

A. Enzymatic Nature - Tissue homogenates and purified enzymes from many sources have been found to hydrolyze ribonucleic acids. However, of the pancreatic enzymes from the numerous species studied, only beef pancreatic ribonuclease has been well characterized. Thus on a comparative basis, a discussion of the enzymic properties of the mouse pancreas ribonucleases will be largely limited to similar studies with the beef enzyme.

The evidence presented in this thesis indicates that beef pancreatic and mouse pancreatic ribonuclease have quite similar catalytic properties. Both enzymes cause a partial breakdown of RNA and split only secondary phosphate esters of pyrimidine-3'-phosphates. Thus the end products resulting from exhaustive digestion of RNA with beef pancreatic ribonuclease are not further broken down when exposed to the mouse enzymes, although a slight hydrolysis of nucleic acid "core" under special assay conditions by mouse pancreas extracts is probably due to the presence of a minute amount of a "purine ribonuclease". The existence of nucleases which differ in specificity from Kunitz' ribonuclease has been demonstrated many times. Jones (79) reported that all four mononucleotides are present in the hydrolysate when yeast ribonucleic acid is treated with a boiled extract of pig pancreas. Bredereck (80) and Bolomey and Allen (81) obtained substantial amounts of purine nucleosides from incubation mixtures of yeast nucleic acids and enzyme preparations from sweet almond meal. Schmidt (34) et al. report that crude spleen and pancreas extracts act on both purine and pyrimidine nucleotide interlinkages. The spleen nuclease preparation of Maver and Greco (15) was shown by Volkin and Cohn (82) to degrade

ribonucleic acids to the 3'-mononucleotides, and Shuster and Kaplan (83) found a nuclease to be present in their preparation of b nucleotidase that hydrolyzes ribonucleic acids completely. Most recently, Davis and Allen (84) have found a phosphodiesterase to be present in beef pancreas which hydrolyses nucleoside-2', 3'-phosphates to the corresponding 2' nucleotide but shows no activity against RNA. In addition, prolonged incubation of a second beef enzyme preparation with yeast RNA yielded traces of the four mononucleotides, which was attributed to the presence of an unknown phosphodiesterase.

In terms of physical properties such as pH optima and general stability to handling and storage, the mouse and beef enzymes also show striking similarities. In addition, the slow diffusion of the mouse ribonucleases through a dialysis membrane over a 24 hour period is compatible with the view that the mouse enzyme, like the beef enzyme, is a relatively small protein.

In spite of these similarities, chromatographically the beef and mouse enzymes appear to be distinct proteins. In support of this view, 70 per cent of the mouse ribonucleases occupy widely separated positions on chromatographic elution diagrams from columns of the cation exchange resin IRC - 50 (XE - 64). The physical differences thus revealed are not entirely species specific, however, since mixtures of the beef and mouse pancreatic enzymes cannot be completely resolved chromatographically; peak II from a Hirs et al. extract of mouse pancreas coincides with the B component from a corresponding extract of beef pancreas.

B. Microheterogeneity of Mouse Pancreas Ribonuclease - Although mouse pancreas extracts demonstrate a chromatographically heterogeneous population of ribonucleases, this cannot be taken as direct evidence of several distinct enzymes in this tissue. It should be recognized that in this respect, homogeneity is a relative concept and must be operationally defined in terms of the method or methods of study used. The fact that one may isolate multiple related proteins does not necessarily prove that these are all bona fide products of protein biosynthesis. Zymogens and their closely related active derivatives, fibrinogen and fibrin, hypertensinogen and hypertensin, trypsinogen and trypsin, are examples in which it seems likely, even though not rigidly established, that the synthetic machinery makes only the precursor molecule and that the products are formed from them subsequently. Furthermore, there is the real possibility that procedures used in isolation and purification may contribute to inhomogeneity. It is important, therefore, at this point, to consider the methodology used in these studies in relation to similar studies and the possible pitfalls involved.

a. Starting material - handling and storage - The homogeneity of the starting material must be considered. For example, Brown, Sanger, and Kitai (85) suggest that the finding in beef insulin samples of small and variable amounts of peptide sequences characteristic of sheep insulin and pig insulin may be attributable to contamination at the slaughter house. Thus the question arises as to whether an often diffuse organ such as the pancreas is appreciably infiltrated by other tissue. In the case of the mouse this does not appear to be so. Mouse pancreas is a discrete rather than a diffuse organ and it is easy to locate and excise,

with limited attachments to the surrounding tissues.

Modification attributable to handling and storage is known to contribute to inhomogeneity in a number of instances. Hirs (quoted in ... Raacke, 86) has shown that chymotrypsinogen, homogeneous on IRC - 50 columns, develops asymmetry on storage. Loss of amide groups has been encountered during purification or storage of several proteins. Raacke has shown that one months storage of lyophilized ribonuclease causes some conversion of the A form to the B form (86). Moreover, it appears that different preparations of crystalline ribonuclease, while they all contain both A and B, can show widely different proportions of the two forms. Tanford and Hauenstein (87), studying a preparation with approximately equal amounts of A and B, have shown that according to titration data, they differ by only one carboxyl group. Attempts to demonstrate other differences failed, including viscosity and light scattering studies. Of particular interest are the observations made with the sensitive differential spectrophotometric technique. They indicate that the phenolic hydroxyl groups in ribonuclease A and B must be identically situated and identically bonded. These results provide an adequate explanation for the presence of two components in crystalline beef ribonuclease and do not require the postulation of differences in the oxidation state of the sulfhydryl groups (88), although active reduction may indeed modify the native molecule and increase the heterogeneity (89). Apparently, then, storage can lead to deamidation of the B component, and different preparative procedures may cause greater or lesser degrees of deamidation. On the other hand, the studies of Hirs, Moore, and Stein (31) on crude extracts, and on extracts exposed to acid-

alcohol for varying periods of time, strongly suggest that some of the B form is resident in the cell. Conversely however, the isolation of two enzymically distinct ribonucleases from liver extracts (19) and from spleen (17) suggests that much more may be involved than one amide grouping. In addition, since the proteins discussed here are almost invariably prepared from large pools of starting material and since genetically related proteins may differ in only minor respects (90), such as the number of amide groups, it is important to note that the samples examined in these mouse pancreas studies are usually prepared from one to three tissues and should indicate, for duplicate studies, any genetically determined abnormalities.

b. Modification during extraction - activation and inhibition - In contrast to the isolation of multiple related proteins, the fact that a chemically homogeneous protein or several chemically homogeneous proteins can be isolated from a tissue does not necessarily rule out the possibility that the tissue produces a broad spectrum of related protein molecules. There may be a high degree of selectivity in the isolation and purification procedures with the "survival of the purest". Occasionally it is feasible to check this possibility by direct examination of crude extracts with a minimum of handling. Thus, the mouse tissue studies have been conducted differentially using both the classical Kunitz and the Hirs, Stein, and Moore acid extraction and a simple sodium phosphate buffer (0.2 M, pH 6.47) extraction procedure. The Kunitz preparation of crystalline pancreatic ribonuclease makes use of the extraction of tissue with ice-cold 0.25 N sulfuric acid for 24 hours or longer (10). Pirotte and Desreux (91) treated guinea pig liver homogenates with sulfuric acid and compared the activity of the fractions obtained subsequently with untreated fractions

prepared in 0.88 M sucrose solution. In all cases, the untreated fractions (nuclei, mitochondria, microsomes, and supernatant) had only 5 to 14 per cent of the activity of the treated fractions. On the other hand, Rabinovitch (92) found no change in the activity of a pancreas homogenate treated with 0.15 N sulfuric acid. Since no careful study has been made on the effect of sulfuric acid on ribonuclease activity of cell fractions or of crude or purified preparations, the experiments presented in Table VIII have been carried out. No significant change was noted in the whole-tissue extracts while a similar acid-treatment of the cellular fractions resulted in a decrease in the ribonuclease of the nuclear fraction and a corresponding increase in the microsomal and soluble enzyme. This observed effect on sulfuric acid treatment may be explained in any of several ways. Sulfuric acid may effect a conversion of one or more of the forms of the ribonucleases present in the cell to related forms or there may be an inactive form of the enzyme which is activated by acid. Conversely, one may postulate a similar mechanism through the selective inactivation or loss of an inhibitor.

Analytical data indicate that each of these explanations may hold for mouse pancreas. In support of the former possibility (i.e., an acid conversion or inactivation of certain enzymes), the following evidence may be presented:

(1) The III_a component of the non-acid treated microsomal fraction (Fig. 9), a III_b component from the non-acid treated nuclear fraction (Fig. 7), and component I of the non-acid treated supernatant (Fig. 11) undergo conversion or inactivation on sulfuric acid treatment.

In the case of the nuclear fraction for example, it is clear that either component III_b or II is largely inactivated, in addition to a partial conversion (see Table VIII).

(2) In addition, analyses of the effluent fractions from a chromatographic study of the non-acid treated supernatant show a corresponding increase (i.e., 455 to 463 per cent) in effluent activity to that found for the same sulfuric acid treated sample (Table VIII). These data suggest that the inactivation or loss of an inhibitor may be the correct one. Thus on treatment by the procedure of Hirs et al., the inhibitor would be inactivated by the sulfuric acid treatment to give an increase in ribonuclease activity, while on chromatographic elution the inhibitor would be selectively separated from the enzyme by the column. A less pronounced increase in ribonuclease activity on sulfuric acid treatment or chromatographic elution of the microsomal fraction suggests that a similar situation may exist in this particulate fraction. In this respect it is interesting to note that Roth (26) has reported that treatment of a rat liver homogenate with 0.25 N sulfuric acid increases the acid ribonuclease activity by about 50 per cent. However, on studies with a limited number of cell fractions, he found acid treatment caused a loss in the acid ribonuclease activity of the mitochondria but this was more than compensated for by increases in the enzymatic activities of the microsomal, nuclear, and supernatant fractions. This observed increase is apparently due to a complex inhibitor inactivation, and activation of latent forms of the alkaline liver ribonuclease. Of a similar nature, de Duve and coworkers (93) have also studied the distribution and properties of ribonuclease in

hepatic cell fractions and found that the activity of the acid ribonuclease, which was located primarily in their heavy and light mitochondrial fractions, could be increased by repeated freezing and thawing, exposure to Triton X-100, or water, and incubation at 37°. In view of these findings, and findings from our own laboratory (94), the studies of latent ribonucleases and inhibitor interactions are not in conflict. An additional question that remains to be answered, however, is does this latent or inactive enzyme actually have a physiological function in the cell, or does it merely represent ribonuclease which has passively diffused from the particulate structures and inactivated as a protective device by the soluble portion of the cell? Further experiments are necessary to answer these and many other questions.

Finally, the question arises as to whether mouse pancreas ribonuclease has been partly degraded during the course of isolation and chromatography. Digestion of beef pancreatic ribonuclease has in fact been shown to lead to the formation of products of lower molecular weight which are still enzymatically active (95,96). However, in the isolation of the mouse pancreas enzymes it has been shown in Table III that all measurable proteolytic activity can be precipitated from the acid extracts of the Hirs et al. preparation before the final pH adjustment is made and the preparation chromatographed. A more rigorous proof of the stability of the pancreatic ribonucleases was made by subjecting an aqueous tissue homogenate to the proteolytic activity present in the pancreas for 6 hours at room temperature. No detectable change in the elution pattern of the Hirs et al. extract was evident. Finally, care was taken to avoid

autolysis in preparing pancreas extracts, and in our view it is very unlikely that the eluted peaks of mouse pancreas ribonuclease are a proteolytic split product.

c. Chromatographic elution analyses on IRC - 50 cation exchange resin - In the interpretation of effluent patterns it should be borne in mind that the position of a peak is more than usually sensitive to several factors. In a single stage process, changes in the composition of the sample put on the column, in regard either to proteins, neutral salts, or other small molecules, may alter the effluent pattern. Thus, it may not be possible to conclude that two mixtures of proteins are the same or different by inspection of the effluent curves. Several types of experiments can be performed to make more certain the interpretation of effluent curves obtained from any type of protein chromatogram. Rechromatography of a given peak to insure that the behavior of a given component when isolated is the same as when in a mixture is extremely important. In this respect, in the mouse pancreas studies, the recovery of both total protein and enzymatic activity on rechromatography has been virtually quantitative (Fig. 4). As a result there is every reason to believe that these experiments represent a multistage process possessing resolving power comparable to that obtained in the elution analysis of solutes of lower molecular weight.

II. Intracellular Localization of Mouse Pancreas Ribonucleases -

It is evident from observations described by numerous workers that conclusions as to the localization of biochemical properties in specific cell structures are in most instances based on the cytological identification of an isolated cell fraction as homogeneous. In the technique of cell fractionation, the cells of the tissue are mechanically disrupted, and the nuclei, mitochondria, and other cellular components are released into a suitable medium from which they can be isolated by differential centrifugation. This method has the advantage of permitting the isolation of all the particulate components of cells from a single sample of tissue in a yield and degree of homogeneity sufficient to allow an accurate comparison of the properties of the isolated structures. However, although it is obvious that the cell fractionation procedure is capable of yielding more information than any of the other cytochemical methods, it is important to define the limitations of the technique.

It is necessary first of all to recognize that tissues do not represent uniform populations of a single cell type. However, with the pancreas, in the predominant cell type of this gland, i.e., the acinar or exocrine cell, the endoplasmic reticulum occupies a greater proportion of the cytoplasmic volume than it does in almost any other tissue. The other cell types present in this gland are the centro-acinar cells and the duct epithelia, the endocrine cells or the cells of the Islets of Langerhans, and the cells of the connective tissue and blood vessels.

Since it is therefore apparent that the non-acinar cells account for a relatively small proportion of the total cytoplasm of the pancreas, the

fact that they comprise less than 5 per cent of the total cell population is of significance only in studies in which the composition of the average acinar cell is calculated on the basis of DNA or of the total number of nuclei present.

A second and perhaps even more important consideration of non-uniformity of cell populations is that as shown by light microscope studies (97,98) that the acini of the pancreas are usually found in different functional stages. Complete synchronization is difficult if not impossible to attain. Apparently, only periods of long fasting followed by feeding will bring a large part of the exocrine cell population into functional synchrony. This specific problem will be discussed later in connection with individual variations in the ribonuclease elution patterns from the isolated cell fractions.

Finally it is necessary to consider the argument frequently used in peremptorily dismissing cell fractionation as a biochemical tool, namely, that at the moment of, or immediately after cell rupture, so many artifacts occur (such as morphological alterations, adsorption, release of particle bound enzymes through autolysis or solubilization by the suspending medium, etc.) that it is utterly useless to isolate the cell structures and study their properties. However, the important criteria should be, that while it is important to realize that such artifacts can occur, it is at the same time necessary to carry out experiments to determine whether they do occur. The following studies on the intracellular localization of mouse pancreas "nucleases" demonstrate that of six chromatographically distinct enzymes, two are present in single fractions and that only one ribonuclease can be demonstrated in as many as three of the ten total fractions. In fact, the

fraction which might be expected to contain the broadest spectrum of ribonucleases, the final supernatant, contains (under certain conditions) a single enzymatically active ribonuclease component, a component localized specifically in the soluble portion of the cell. Thus, at least in the case of the enzyme ribonuclease, very little release of particle bound enzyme and readsorption would seen to have occurred.

According to their intracellular distribution pattern, the mouse pancreas ribonucleases studied can be divided into at least four groups, which are believed to correspond to at least three distinct classes of cytoplasmic granules.

A. RNase Activity in the Nuclear Fraction - The first group comprises the enzymes of the nuclear fraction, and accounts for nearly 20 per cent of the total cellular ribonuclease activity. These enzymes appear to be largely associated with heavier cytoplasmic granules rather than nuclei, since an in vitro pilocarpine stimulation causes the secretion of a corresponding chromatographic elution profile of "nuclease" activity. The key to the identification of this specific fraction lies in the localization of a specific sulfuric acic-labile peak III component in the nuclear fraction and not in the principle zymogen fraction (Figs. 7 and 8). It is thus indicated that two types of secretory granules are present in the exocrine cell, one consisting of a very dense population and sedimenting with the nuclei and actively extruded under stimulation by a parasympathomimetic agent, and the second sedimenting in a fraction designated as the "principle zymogen fraction", and released under hormonal stimulation.

However, the ubiquitous occurrence of ribonuclease activity in all types of cells capable of reproduction, which have thus far been examined

for the presence of this enzyme, suggests that these "nucleases" may play an important role in the growth and development of the cell. Such a function might implicate a nuclear ribonuclease, and therefore necessitates chromatographic studies similar to those discussed above be carried out on purified nuclei preparations to establish the actual level, if any, of a nuclear enzyme.

B. RNase Activity in the Zymogen Granule Fraction - The second group of "nucleases" comprise the enzymes of the so-called "principle zymogen granule fraction". In the usual fractionation scheme, this fraction occupies an intermediary position between the nuclear fraction and mitochondrial fraction and thus must consist primarily of intact zymogen granules and heavy mitochondria. However, the fraction thus obtained accounts for only 5 to 10 per cent of the total ribonuclease activity of the pancreatic homogenates, although it has the highest specific activity of any of the other cell fractions. The ribonucleases localized in these particles are the II components of both acid-treated and non-acid treated zymogen extracts (and also present in the nuclear fraction), an acid-stable component from peak III, and possibly a small part of the acid-labile peak III component as well, overlapping from the nuclear fraction.

It is interesting to note, that on the basis of the distribution of the ribonuclease activity between the nuclear and zymogen fractions, it would appear that secretory granules are found in highest concentration in the nuclear fraction. In contrast, the amylase activity has been shown to be nearly two times higher in the intermediate zymogen fraction than in the adjacent nuclear or mitochondrial fractions (73). In view of these

results, it can be suggested that the zymogen granules are biochemically a heterogeneous population in which one enzyme (ribonuclease) is associated with the denser, heavier secretory granules while a second enzyme (amylase) is correspondingly found in a lighter zymogen fraction.

The overall localization of these hydrolytic enzymes in the zymogen granules is in agreement with a number of workers. Experiments by Siekevitz and Palade (46) have shown, in fact, that the zymogen fraction of guinea pig pancreas contained about one-third of the ribonuclease activity of the whole homogenate and in agreement with our data had the highest specific activity of the cell fractions. In addition, they also concluded that a large part of the trypsin-activatable proteolytic enzymes (i.e., mainly trypsinogen and chymotrypsinogen) produced by the pancreatic exocrine cells is localized in the zymogen granules. Hokin found that, in addition to proteolytic activity, the zymogen granules show lipase and amylase activity (41) at concentrations higher than those of the whole homogenates. In contrast, according to Laird (99) most of the amylase activity is recovered in the microsome fraction. In her experiments, however, a distinct zymogen fraction was not isolated.

It appears then, from the results of Hokin, Siekevitz and Palade, and from our own in collaboration with Van Lancker, that the five hydrolytic enzymes or enzyme precursors, namely chymotrypsinogen, trypsinogen, procarboxypeptidase, amylase, and lipase are concentrated to an appreciable extent in the granules sedimenting with or immediately after the cell nuclei. Since they represent the main pancreatic enzymes known to be secreted for digestive purposes, it can be proposed, as already implied

by Schucher and Hokin (59), that the part of the ribonuclease which is located in the zymogen granule fractions is also a digestive enzyme produced for excretion into the duodenum. That this indeed is the case has been shown even more conclusively in connection with the chromatographic data from the isolated cell fractions and the secretory products of *in vitro* stimulation.

Finally, the apparent heterogeneity of the nuclear and zymogen fractions poses several interesting and important questions. In the first place, would such a finding mean that the granules in each cell show varying degrees of differentiation or that the granules in a single cell differ from those in neighboring cells? The answers to questions of this type obviously would depend upon methods more refined than the present cell fractionation technique. If differences do exist between the zymogen granules in the same or adjacent cells, one might almost have to study this possibility on the basis of individual cells or even single granules.

With the present scheme of fractionation, the mitochondria are distributed unevenly over four different fractions (i.e., fractions a, b, c, and d). It is of some significance that these subfractions have not brought to light any evidence of localization of ribonuclease activity in the mitochondria. In this respect, the distribution of ribonuclease activity among the pancreatic cellular fractions is noticeably different from that revealed by similar methods in the case of liver. Schneider and Hogeboom (100) found, for instance, that the ribonuclease activity of mouse liver was almost entirely recovered in the mitochondrial fraction, while the microsomes and final supernatant showed respectively little and

negligible activity. They tentatively ascribed the microsomal activity to adsorbed enzyme. By using a more critical fractionation, de Duve et al. (93), arrived at the conclusion that in the liver cells of the rat the ribonuclease is located (together with a whole series of other hydrolytic enzymes) in granules smaller than, and distinct from, mitochondria, for which the name "lysosomes" was proposed. The morphological and biochemical identification of the lysosomes is uncertain, but the structure of the putative bodies demonstrated by de Duve and Novikoff (101) in this same tissue is noticeably different from that of the zymogen granules in the pancreas. Finally, in the kidney, Strauss (102) found the same series of hydrolytic enzymes, ribonuclease included, concentrated in dense "doplets" (0.1 to 5 μ) apparently distinct from the mitochondria.

C. RNase Activity in the Microsomal Fraction - The third group of ribonucleases includes those found in the microsomal fractions. As shown in Fig. 9, there are strong grounds for the belief that the peculiar distribution of ribonuclease reflects the existence of a distinct class of granules in the microsomal fractions. According to the data reported in this thesis, the ribonuclease activity in the first microsomal sub-fraction is, by orders of magnitude, 7 to 10 times that of the two subsequent microsomal sub-fractions, and 4 to 5 times that in the nuclear and zymogen fractions. Since the microsomes are not contaminated by zymogen granules, it is assumed that these findings reflect either the situation in vivo or are the result of enzyme adsorption on the microsomal membranes. The last alternative presupposes that a number of zymogen granules disintegrate during fractionation and liberate hydrolytic enzymes, which might

be subsequently adsorbed on other cytoplasmic structures or remain "solubilized" in the final supernatant. However, one may rule out this latter possibility, to a large extent at least, on the basis of the extremely high relative ribonuclease activity in this microsomal fraction and on the chromatographic elution evidence of a unique distribution pattern in this specific fraction (Fig. 9). The fact that other enzymes (amylase, acid phosphatase, and deoxyribonuclease) in this same class of hydrolytic enzymes are localized in this microsomal sub-fraction (73) provides additional support for this interpretation.

The solution to the problems raised as to the cytological nature and function of these "heavy" microsomes must await the correlation of changes in these granules with physiological changes in the pancreas. Their enzymatic equipment suggests that they may be concerned with localized phenomena of intracellular catabolism and may have little direct bearing on the major synthetic and energy producing metabolic processes. However, with the current emphasis on the possible role of the microsomes in protein synthesis, this concentration of hydrolytic enzymes in this fraction may actually indicate the initial site of synthesis of these enzymes, or alternately, may indicate a much more complex cellular function for these enzymes than previously supposed.

D. RNase Activity in the Cell "Soluble" Fraction - The fourth and final group comprises those ribonucleases present in the soluble portion of the cell and accounts for about 15 per cent of the total cellular ribonuclease activity. As pointed out before, part of these activities undoubtedly originate from damaged granules, but it is probable that the greater

percentage of the activity pre-existed in soluble form in the intact cell. In view of the results presented in Figs. 10 through 13, it is clear that one ribonuclease, peak I from the non-acid treated tissue, is specifically localized in the soluble portion of the cell. The variable amounts of other ribonuclease components cannot be explained at present. Whether these diffusible elements of the cytoplasm represent rapidly turning over precursor "nucleases" from tissues in different stages of the resting phase of the secretory cycle or yet still another reason, will have to await the results of more definitive experiments.

III. Physiological Inhibitors of Cellular Ribonucleases -

In the present study, indirect evidence for one and possibly two ribonuclease inhibitors have been presented. Such evidence is by no means unique, for ribonuclease inhibitors appear to have a widespread occurrence (7), but their physiological role is unknown at present. In the case of the latent ribonucleases of the cell supernatant and to a lesser extent of the microsomes, the question as to whether the inactive or inhibited ribonuclease of the cell is ever physiologically active during the life of the cell is an interesting one. One might conjecture, at least in respect to the secretory granules of the pancreas, that an inhibitor might be essential in the inactivation of the storage enzyme (thus forming a zymogen) and prevent the intracellular digestion of the RNA rich reticular endothelium. In this respect, it has been suggested by Roth (103) that in rat liver the inhibitor functions to inactivate any ribonuclease that diffuses out of the mitochondria or lysosomes during the life of the cell. This

could occur since alkaline ribonuclease is probably a small molecule capable of diffusing through membranes under certain conditions (104). However, if this hypothesis is correct, one might expect a fairly steady increase in the amount of inhibited ribonuclease during the aging of the organism.

It is also possible that ribonuclease inhibitor functions as the rate controlling step in cellular protein synthesis. If RNA serves as a template or the transmitted of genetic information in protein biosynthesis, then a release of ribonuclease from its inhibitor would make the enzyme available to degrade or remove the nucleic acid template from the newly synthesized protein molecule and allow subsequent reformation of the template and further protein synthesis. Total or selective inhibition of ribonuclease would thus control the rate of protein synthesis. Considerable additional work must be done to study ribonuclease inhibitors and inhibited ribonuclease during various physiological changes in the organism before the question of the function can be answered.

IV. Physiological Changes in Mouse Pancreas and the Effect on the Intra- and Extracellular Ribonucleases -

The external secretion of the pancreas *in vivo* is regulated by both hormonal and nervous mechanisms. In the present investigations, the secretory response to the hormone pancreozymin and to the parasympathomimetic drug pilocarpine have been compared in an *in vitro* system. Stimulation of the pancreas by pilocarpine causes a secretion rich in enzymes. This is a cholinergic effect which is blocked by atropine (105) and is presumably mediated by the anticholine esterase action of

pilocarpine. Like pilocarpine, the hormone pancreozymin also stimulates the release of stored enzymes from the exocrine cells of the pancreas, but with little known as to the probable mechanism of action.

It might be appropriate to raise at this point several questions which deal with the secretory cycle and the "active extrusion" of the digestive enzymes of the pancreas, namely:

1. What structural and functional features differentiate the dense secretory granules of the nuclear fraction from those in the principle zymogen fraction? In this regard it will be recalled that the evidence presented indicates that on one hand hormonal stimulation selectively releases the ribonuclease activity of the principle zymogen fraction while in the second instance pilocarpine stimulation selectively releases the corresponding enzymes of the nuclear fraction. The best that can be said at this time is that since under physiological conditions, the hormonal and nervous mechanisms come into play simultaneously, the question of a synergistic or additive effect would be an important efficiency consideration in the release of the digestive enzymes of the pancreas.

2. It is now commonly accepted that the digestive enzymes of the pancreas are present in the cell as stored forms in the secretory granules. However, do these granules then release the enzyme by some unknown mechanism either by extrusion through the cell wall as granules which are immediately solubilized in the duct fluid or by first breaking up in the cell and the enzymes passing through the cell wall as zymogens or free enzymes. Support for this latter possibility is suggested by the extensive release of ninhydrin positive material from the soluble portion of the cell into the incubation medium. As shown in Figure 21, 90 per cent of the ninhydrin

positive material released into the incubation medium on stimulation can be eluted in a very sharp, symmetrical peak at twice the hold-up volume from a column of the cation exchange resin IRC - 50. Furthermore, this represents a secretion of more than 98 per cent of such material contained in the combined extracts of both incubated tissue and medium. However, despite this extensive release into the incubation medium, the tissue levels of the ninhydrin positive material remained about the same. Thus the 100 fold increase in the ninhydrin material of the incubation medium must be apparently due to an extensive synthesis and/or release of this material inside the secretory cell, and suggests a selective release of soluble cell components during active extrusion of the hydrolytic enzymes.

These results are not without support from other workers. The findings obtained by Lin and Grossman (106), and by Kalser and Grossman (107) tend to favor the view that storage may be important for the initial discharge of large amounts of enzymes but is not indispensable for the secretion of high concentrations of enzymes. After several hours of repeated stimulation with large doses of parasympathomimetic drugs, the acinar cells have been found to be practically depleted of zymogen granules (108), and yet the cells still respond in a quantitative manner to appropriate stimulation. This latter fact suggests that there is a "dry" secretion of the digestive enzymes at the onset, consisting of the enzymes contained in the zymogen granules, but when the flow of alkaline juice becomes established the enzymes are formed and secreted dissolved, without appearing as granules.

V. On the Intracellular Status and Function of Mouse Pancreas Ribonucleases -

A. Intracellular Status - The results presented in this thesis show clearly that particular "nucleases" are situated in particular fractions, but the interpretation, especially of small activities, is made somewhat difficult by the possibility of mutual contamination of the different fractions and the fact that, since the number of fractions may be much smaller than the number of different cell structures, some of them must contain several different kinds of structures. Contamination may be due to adsorption, to adhesion, to aggregation of smaller particles, to autolysis or the extraction of components, or to other factors.

On the basis of effluent recoveries and stability to acid treatment*, it may be suggested that the enzymatic picture presented by the chromatographic elution pattern from the sodium phosphate buffer pancreas extract more nearly represents that of the "native" intracellular ribonucleases. If this assumption can be made, then it is apparent, within the limitations of the techniques used, that at least six distinct ribonucleases exist in the secretory cell of the pancreas. These enzymes may be outlined as follows:

1. An acid-labile* component eluting at 10 ml. and present only in the soluble portion of the cell (Figs. 10 to 13).

2. The front-running component of peak II, probably acid-stable, and seemingly present, to a small extent at least, in all of the cell fractions studied. The uniqueness of this component can only be

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* For definition of acid-stable and acid-labile see text under "Results".

clearly seen in certain supernatant preparations (Figs. 12 and 13), and apparently is largely localized in the soluble portion of the cell.

3. The slower eluting component of peak II. Like 2 above, this "nuclease" is probably acid-stable and can be clearly demonstrated in certain supernatant preparations. However, unlike 2, this component appears to be uniformly and universally distributed throughout the fractions studied.

4. The front-running acid-labile component of peak III. This enzyme appears to be located exclusively in the cytoplasmic f microsomal fraction (Fig. 9) and accounts for over 70 per cent of the total activity of this fraction.

5. The slower eluting acid-labile component of peak III, localized (as far as can be determined) principally in the heavy granules sedimenting with the nuclei (Fig. 7). This enzyme appears to be largely inactivated by treatment with strong acid although it may be partially converted into a component eluting with peak II.

6. Finally, the slowly eluting acid-stable component of peak III. This enzyme accounts for at least 50 per cent of the ribonuclease activity of the "principle zymogen granule fraction" (Fig. 8), as well as appearing in the cytoplasmic f microsomal fraction (Fig. 9), and occasionally to a small extent in the final supernatant (Figs. 10 to 13).

It is evident then, that the fractions isolated by differential centrifugation from pancreas homogenates are enzymatically heterogeneous at the resolution level of column chromatographic methods, and that the heterogeneity is most pronounced in the cytoplasmic f microsomal fraction. It is also apparent, however, that within a given fraction this heterogeneity is reproducible, with the notable exception being the final supernatant.

B. Intracellular Function - Ribonuclease activity appears to have a very widespread occurrence throughout the plant and animal kingdoms. In fact, different tissues from the same animal often contain different ribonucleases, and a wide variety of enzymes hydrolyzing nucleic acids have been isolated from plant and animal sources. It is interesting, therefore, to conjecture as to the physiological role of these enzymes within the cell.

Thus for the adult pancreas, a number of hypothesis may be advanced to explain why structurally or enzymatically different ribonucleases appear in different proportions in the tissue extracts.

An obvious function of the intracellular ribonuclease of mouse pancreas, or of the pancreas of any species, is one of a storage enzyme in the secretory granules for release into the duodenum to aid in the digestive processes. In this sense, it might be suggested that certain of these enzymes represent precursor forms of ribonuclease activity, perhaps at the point of synthesis, or perhaps undergoing changes in the maturing zymogen granules in various stages of the resting phase of the secretory cycle.

Of a more general nature, it has been suggested by Heppel and coworkers (5), and Heppel, Whitfeld, and Markham (6), that the existence of these enzymes in cells may well be for the purpose of carrying out synthetic reactions rather than for effecting hydrolyses. In one sense, neither ribonuclease or the various phosphodiesterases appear to be able to synthesize phosphodiester bonds de novo. Ribonuclease, however, would appear to possess a unique mechanism for the preservation of such structures by means of the conversion of the normal type of internucleotide bond into

the analogous intranucleotidic cyclic 2':3'-phosphate diester bond, which the enzyme is then able to reconvert into a diester bond of the more usual type. A mechanism of this type is clearly able, in theory at least, to take part in reactions involving rearrangements in RNA's, and while it has been shown that such reactions can only take place among relatively small polynucleotide chains, there would appear to be no a priori reason why it should be restricted to such simple compounds.

It is also possible that the enzyme ribonuclease plays an important part in the changes that take place during cell division. Jacobson and Webb (109) have demonstrated histologically an RNA or ribonucleoprotein coating on the chromosomes which disappears during certain stages of cell division. In addition, the division process coincides with a large increase in the sulfhydryl content of the cell (110). In the liver, a release of ribonuclease from an inhibitor has been shown to give rise to free -SH groups (103); the released ribonuclease would then be available to degrade the RNA or the ribonucleoprotein of the chromosomes.

Of the many theories that have been proposed concerning the mechanism of protein synthesis, the one proposed by Roth (111) involving the interaction of ribonuclease activity is quite interesting. If one presumes that ribonucleic acid acts as a template upon, or alongside which, the amino acids in the peptide chain are supposedly regulated by the highly specific arrangement of the nucleotides which then, as has been suggested, effect peptide bond synthesis, it is not clear how the peptide chains, once formed, would be separated from the nucleic acid. It is possible, however, that the release of the protein may be accomplished by partial

or complete degradation of the polynucleotide chain by intracellular ribonucleases.

In conclusion, it is obvious that these studies present a crude picture of an intracellular enzyme system, a possible lead toward a first approximation at best. However, there is no reason to assume that the problem is "limitless", since the level of significance, the depth to which one can penetrate into a problem, is only limited by methods. Thus the rapidly increasing information about "nuclease" reactions and systems will be accompanied by attempts to apply this knowledge to the intact cell, the ultimate goal being an understanding of the processes which determine the integration of enzymatic activities on the subcellular level.

SUMMARY

1. The evidence presented in this thesis indicates that mouse pancreas ribonuclease and beef pancreas ribonuclease have quite similar catalytic properties. Both enzymes cause a partial breakdown of RNA and split only secondary phosphate esters of pyrimidine-3'-phosphates. Further, both enzymes have similar pH optima and general stability to storage and handling.

2. Mouse pancreas ribonuclease can be chromatographed on columns of the carboxylic ion exchange resin IRC-50 (SE-64). When 0.2 M sodium phosphate buffer, pH 6.47, extracts of mouse pancreas are prepared and chromatographed, the enzymatically active material was eluted in two broad zones. However, when 0.25 N sulfuric acid extracts of mouse pancreas are chromatographed, two sharp, distinct peaks of ribonuclease activity can be recognized, indicating that an alteration of the protein molecule occurred during the acid treatment. pH 7.5:5.0 ribonuclease activity ratios for the individual fractions indicate an enzymatic as well as chromatographic heterogeneity both within a given peak as well as between the peaks from a sulfuric acid extract of mouse pancreas. These chromatographic elution components have been further studied by limited rechromatography and under conditions of pH gradient elution.

3. The intracellular distribution of the ribonucleases of mouse pancreas was studied by differential centrifugation of pancreas homogenates prepared in 0.25 M sucrose. Approximately 70 per cent of the ribonuclease activity in the tissue was associated with three (of one nuclear and nine cytoplasmic) isolated cell fractions. The highest level of ribonuclease activity was found in the "cytoplasmic f" microsomal fraction (35 per cent), with lesser amounts being found in the nuclear dense granule fraction (20 per cent) and the cell "soluble" or supernatant fraction (15 per cent).

4. The effect of treating the nuclear and cytoplasmic pellets isolated from mouse pancreas homogenates with 0.25 N sulfuric acid by the method of Hirs et al. was studied. Large decreases in the activity of the nuclear fraction as well as moderate increases in the "cytoplasmic f" microsomal fraction was observed, and the significance of these changes are discussed.

5. The cell supernatant contained a significant level of ribonuclease activity when the tissue was homogenized in sucrose, and this level could be increased four or five fold by treatment with a strong acid or by column chromatography. This inactive of "latent" ribonuclease may be bound to a protein inhibitor which is removed by an acid-inactivation or by chromatography. The relationships of these findings to other studies on cellular enzyme inhibitors are discussed.

6. Chromatographic elution studies were carried out on both the 0.2 M sodium phosphate buffer extracts and the 0.25 N sulfuric acid extracts of four ribonuclease "rich" cell fractions. Differential analyses of the elution patterns from these cell fractions indicate, based on stability or lability to acid treatment and intracellular localization, that at least six distinct forms of the enzyme may be present in the 0.2 M sodium phosphate buffer extracts of mouse pancreas. Further, two of the six ribonucleases have specific intracellular locations, and only one of the six enzymes appears to be present in each of the fractions investigated.

7. Mouse pancreas has been incubated in vitro with a parasympathomimetic drug (pilocarpine) and with the hormone pancreozymin. Secretion (active extrusion) of both amylase and ribonuclease and an increase in respiratory rate occurred during incubation. Large differences in both the stimulated and non-stimulated extrusion of enzyme were found in different strains of mice. The means of killing the animals and pretreatment of the tissue also affected secretion.

Chromatographic analyses of the ribonuclease "actively extruded" into the incubation medium by maximal in vitro stimulation with pilocarpine or pancreozymin suggests the following hypothesis:

The enzyme associated with the nuclear fraction is contained in dense secretory granules which are selectively released on in vitro pilocarpine stimulation. In contrast, a less dense secretory granule, present in the so-called "principle zymogen fraction" of the cell, is selectively released by in vitro pancreozymin stimulation. In addition, the appearance

of very large amounts of ninhydrin positive material (normally present almost exclusively in the soluble portion of the cell) in the incubation medium further suggests an involvement of the cellular cytoplasm in the secretory mechanism.

9. The cytochemical implications of the intracellular status and function of mouse pancreatic ribonuclease and the relationships of these findings to previous studies on cellular ribonucleases are discussed.

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APPENDIX

Duplicate Chromatographic Elution Studies to Figures 2a, 3, 7, 8, 9, 18, and 21a of the Results section. See reverse side of page 36.

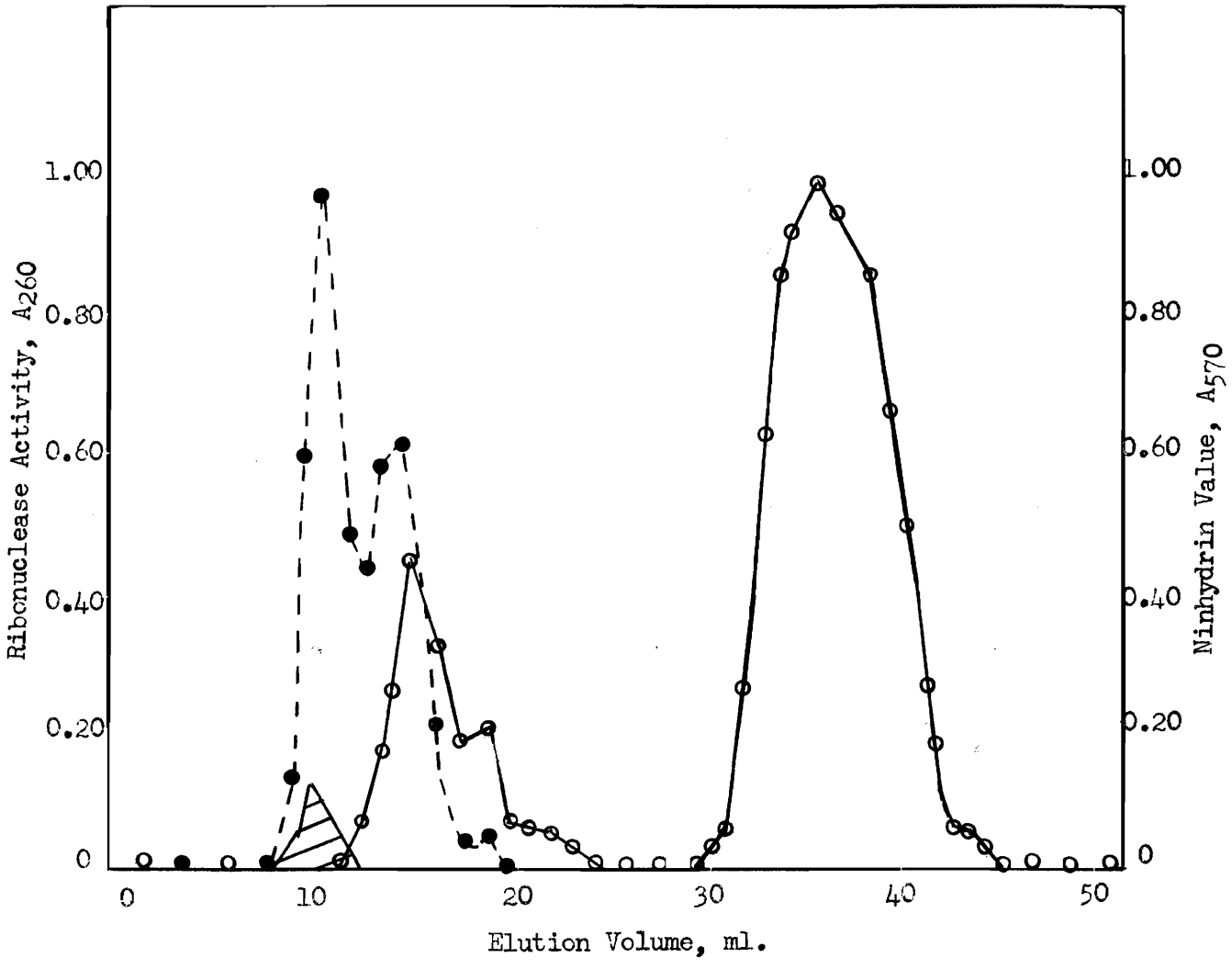


Figure 2A. Chromatography of a 0.25 N sulfuric acid extract of mouse pancreas on a 0.9 x 30 cm. column of IRC-50(XE-64), with 0.2 M phosphate buffer, pH 6.47, as the elution agent. a, effluent volumes assayed for ribonuclease activity at pH 5.0. For details of the procedure see the text under "Methods". ●, ninhydrin value; O, ribonuclease activity. Shaded area 260 mμ absorbing component.

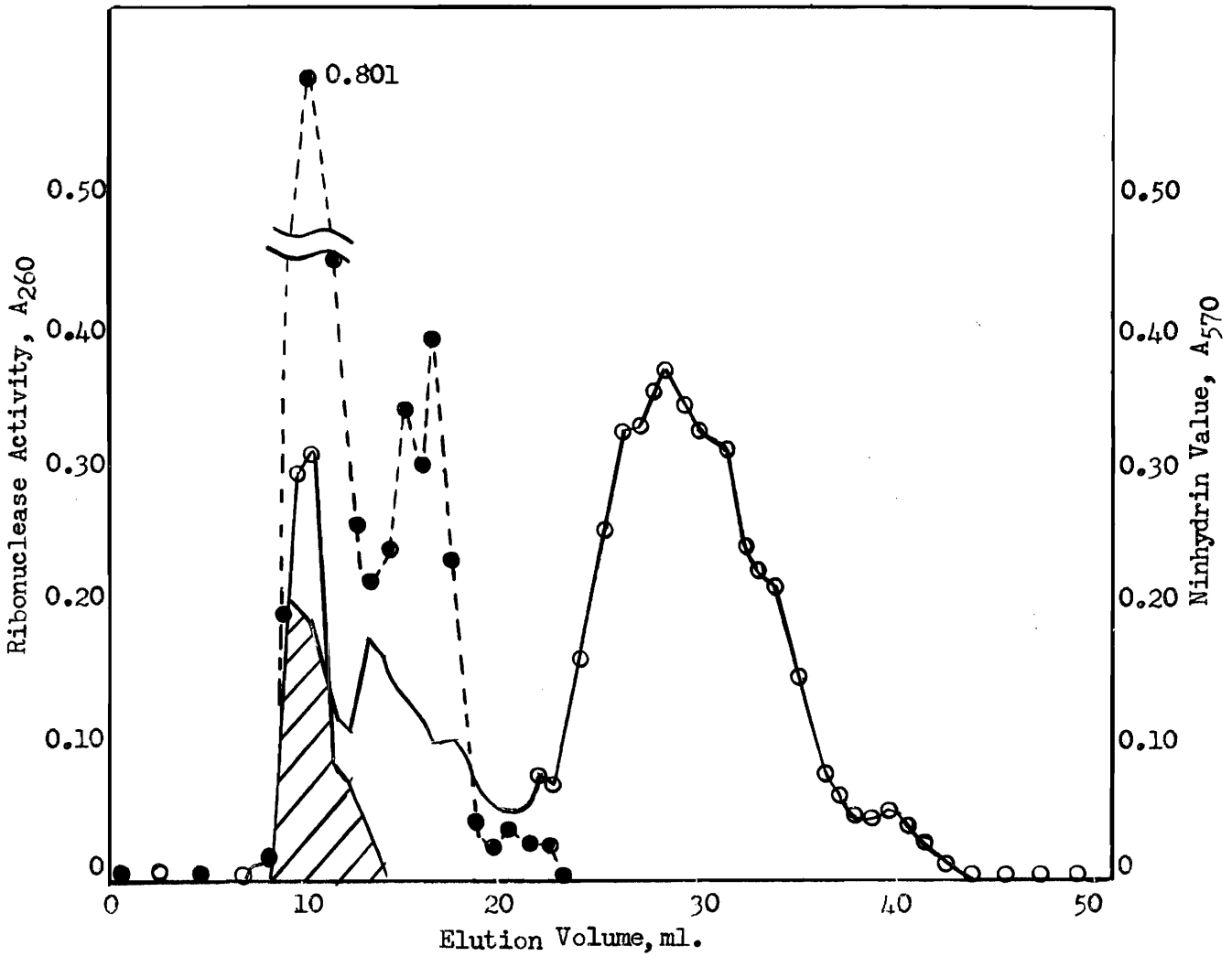
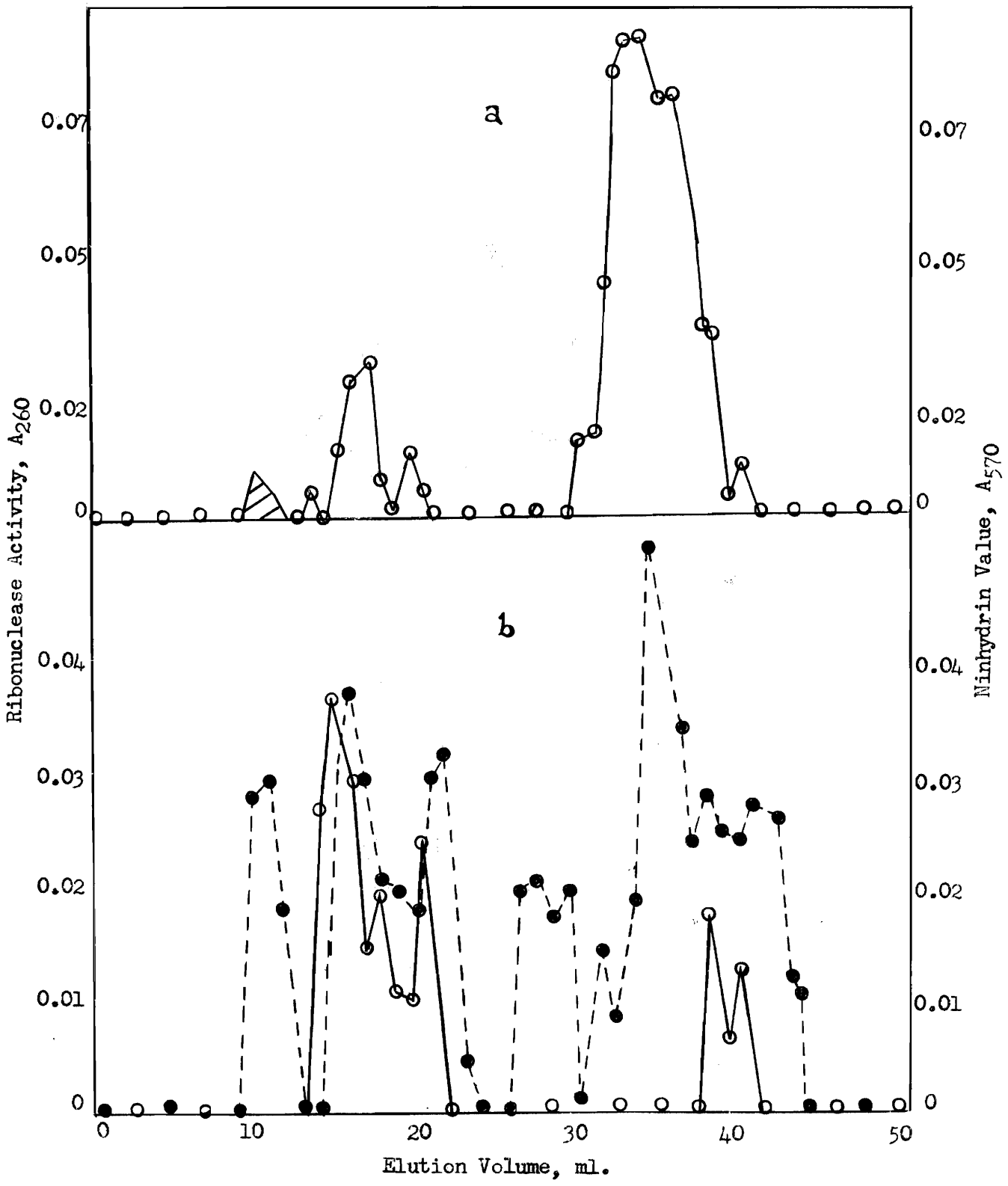
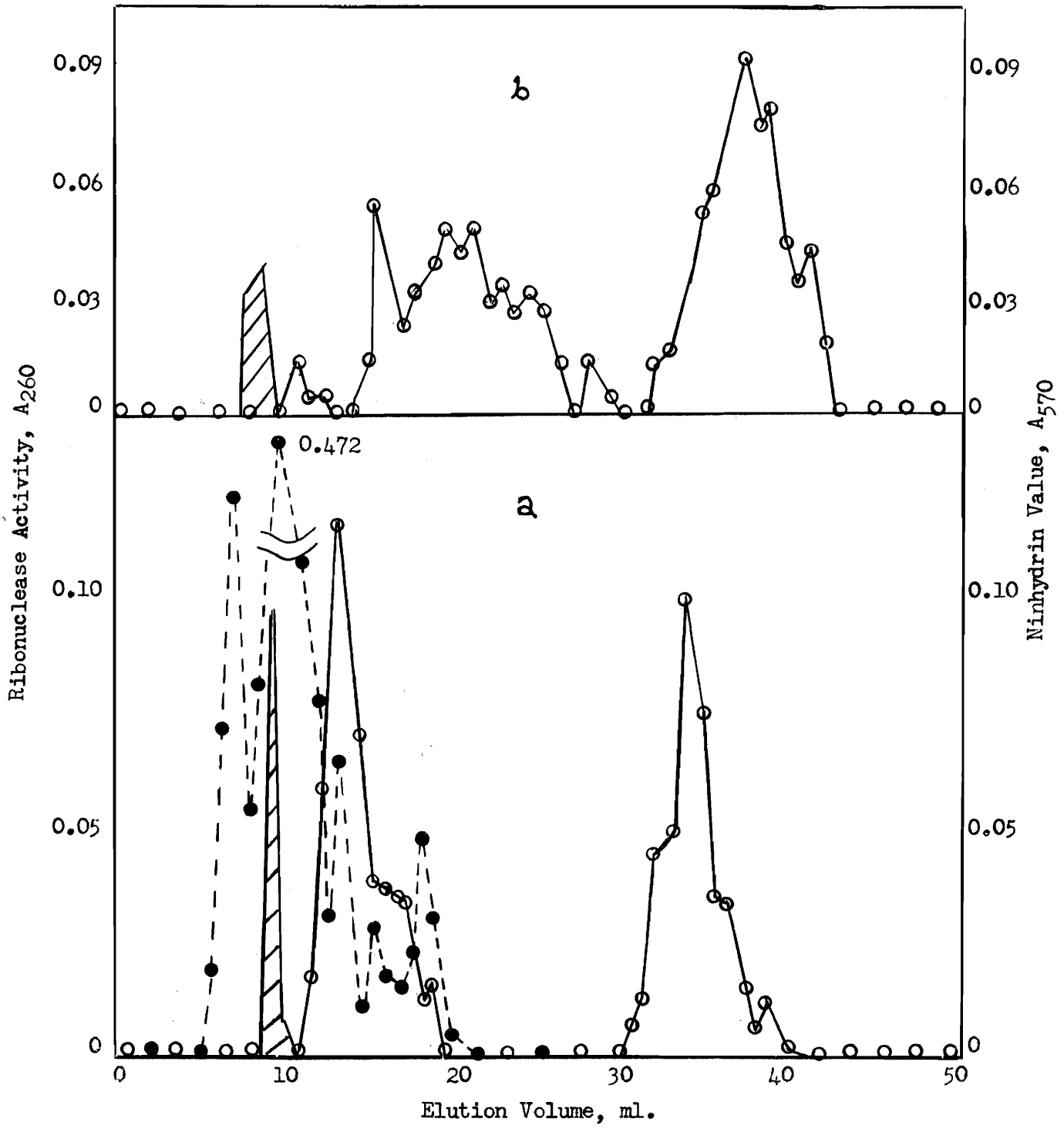
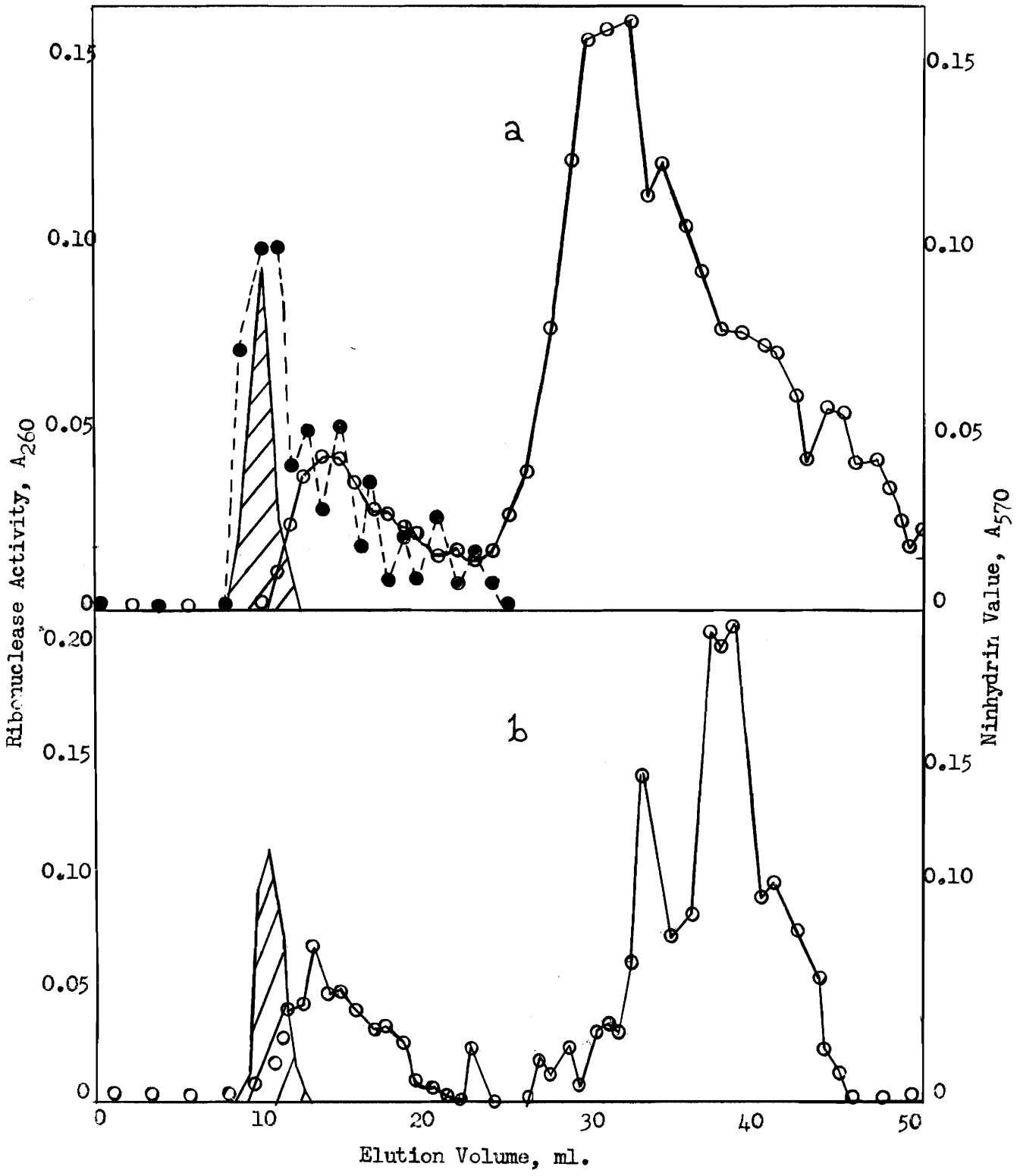
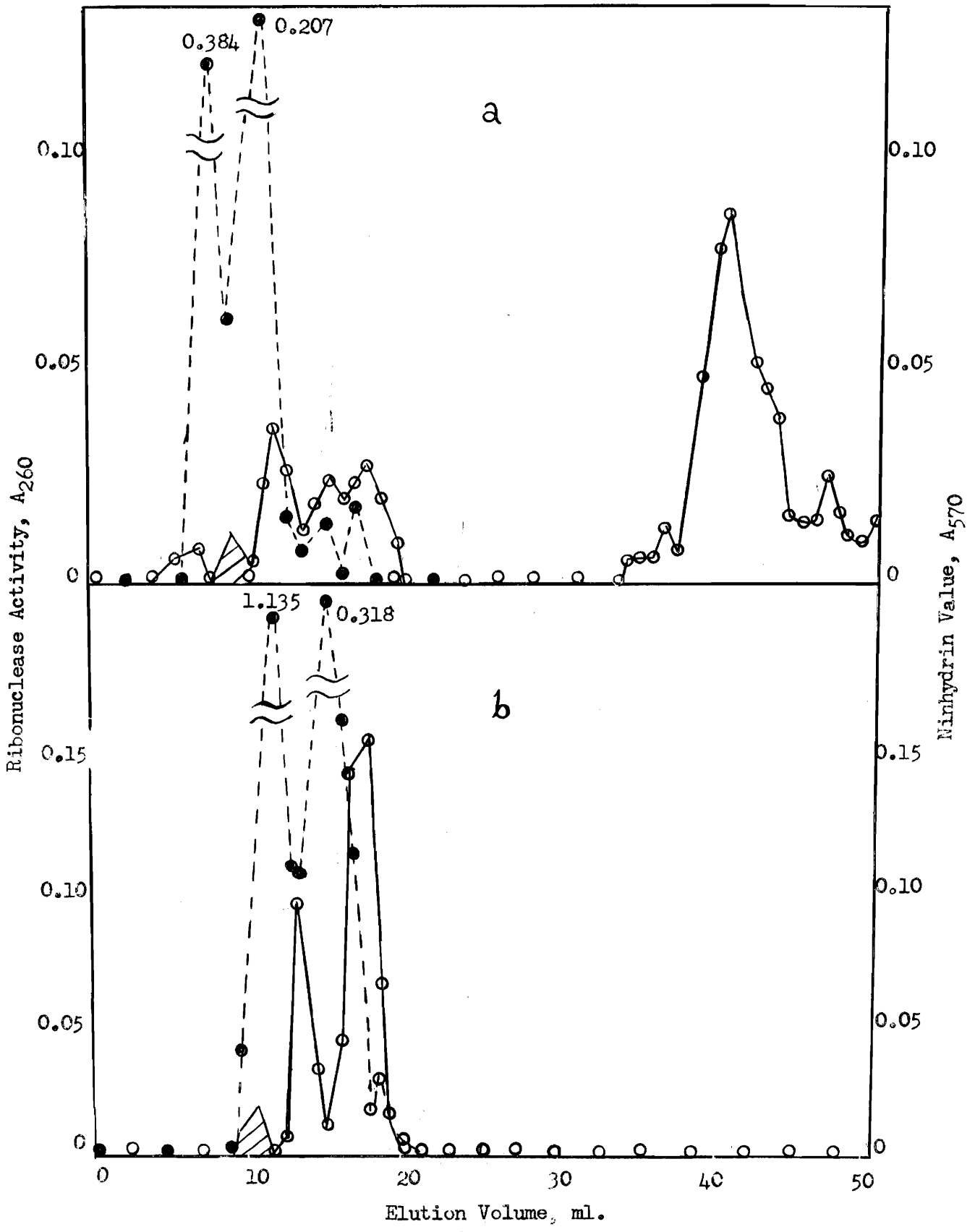


Figure 3A. Chromatography of a sodium phosphate buffer extract (0.2 M, pH 6.47) of mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer, pH 6.47, as the elution agent. Effluent volumes assayed for ribonuclease activity at pH 5.0. For details of the procedures see text under "Methods". ●, ninhydrin value; O, ribonuclease activity measured at pH 5.0. Shaded area, 260 mμ absorbing component.


a
b
0.07
0.05
0.02
0
0.04
0.03
0.02
0.01
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Elution Volume, ml.


b
a
0.472
0.09
0.06
0.03
0
0.10
0.05
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Elution Volume, ml.


a
b
0.15
0.10
0.05
0
0.20
0.15
0.10
0.05
0
10
20
30
40
50
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}
Elution Volume, ml.


0.384
0.207
a
0.10
0.05
0
1.135
0.318
b
0.15
0.10
0.05
0
0
10
20
30
40
50
Elution Volume, ml.
Ribonuclease Activity, A_{260}
Ninhydrin Value, A_{570}

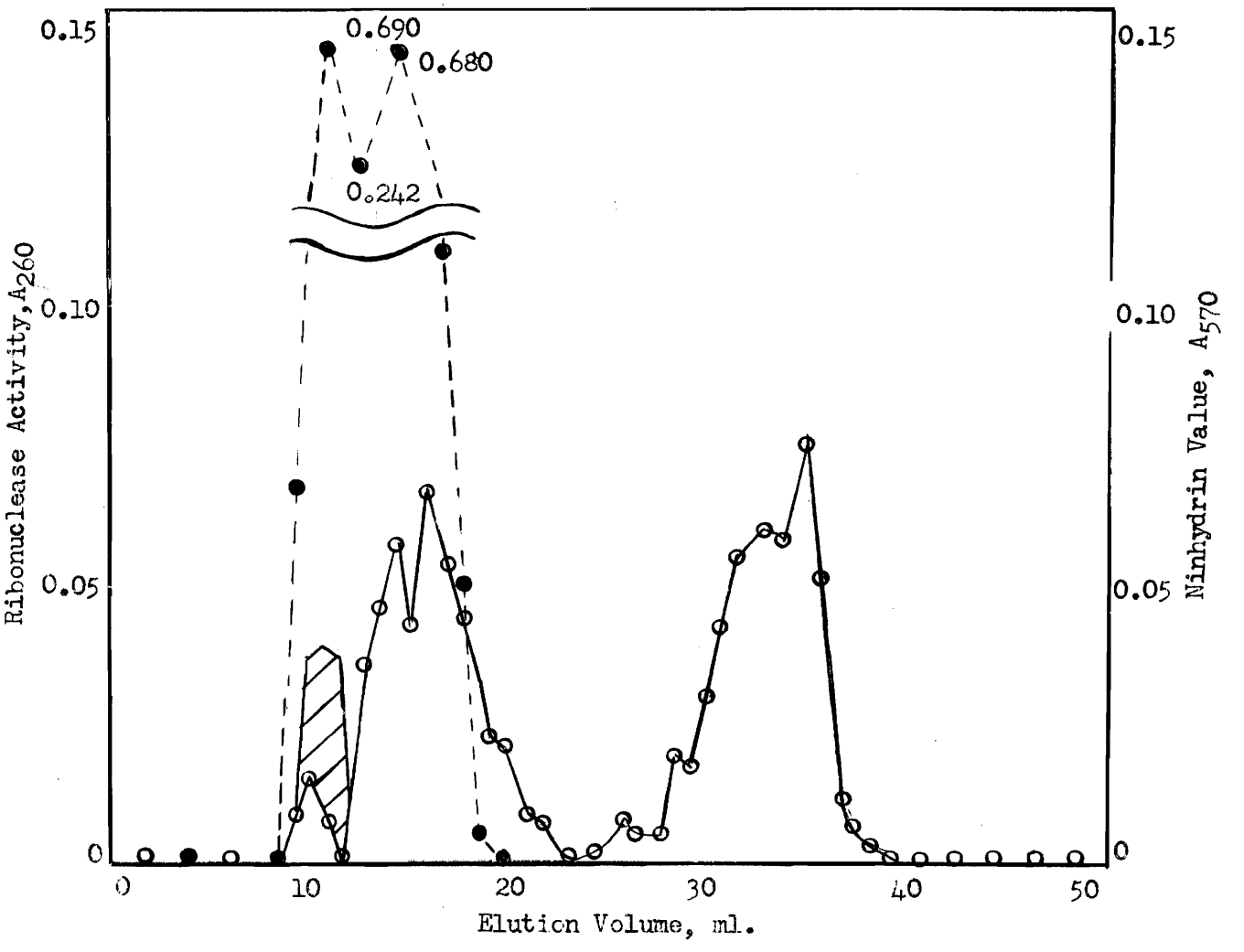


Figure 21A. Chromatography of 0.25 N sulfuric acid extracts of *in vitro* stimulated mouse pancreas on a 0.9 x 30 cm. column of IRC-50 (XE-64), with 0.2 M sodium phosphate buffer at pH 6.47 as eluent. Curve obtained from pancreas stimulated with 5 x 10^{-7} M pilocarpine; ●, ninhydrin value; ○, ribonuclease activity assayed at pH 5.0. Shaded area, 260 mμ absorbing component.